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UMI
BIOCHEMICAL AND MOLECULAR STUDIES OF THE SEED COAT OF

BRASSICA CARINATA (A. Braun.) AND OTHER BRASSICACEAE

A Thesis Submitted to the College of Graduate Studies and Research
in Partial Fulfillment of the Requirements for the Degree of
Doctor of Philosophy
in the Department of Plant Sciences
University of Saskatchewan
Saskatoon, Canada

By
Mary Ann Susan Marles
2001

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Abstract

Biochemical and molecular studies were undertaken to identify the basis of differences in seed coat pigmentation between yellow- and brown-seeded members of the Brassicaceae, and in particular, near-isogenic yellow- and brown-seeded lines of Brassica carinata developed from an Ethiopian accession, PGRC/E 21164.

Histochemical staining of dissected seed coats from six genera of the Brassicaceae revealed condensed tannin (proanthocyanidin) and phlobaphene pigments in dark-seeded species but only in scattered spots in most yellow-seeded species. Anthocyanins were not detected in any seed coat tissue of the Brassicaceae species. In leaf tissue, anthocyanin content was lower in yellow-seeded B. carinata at the three- to four-leaf stage grown at 20 to 25°C, compared to similarly grown brown-seeded material. At cooler temperatures (15 to 18°C), yellow- and brown-seeded lines produced similar amounts of anthocyanin in leaves at the three- to four-leaf.

In chromatographic analyses (TLC, HPLC), phenylpropanoid and flavonoid metabolic intermediates, t-cinnamic acid, dihydroflavonols (dihydromyricetin [trace amounts], dihydroquercetin and dihydrokaempferol) and flavonols (quercetin, kaempferol) were more abundant in hydrolyzed, methanolic extracts of seed coat tissue of yellow-seeded B. carinata than in extracts from seed coat tissue of the brown-seeded B. carinata or brown-seeded B. carinata cultivar S-67. Myricetin was not detected in any seed coat extracts of B. carinata. Mass spectra were generated for phenylpropanoid and flavonoid aglycones from seed coat extracts and authentic
standards. Novel identification techniques were discovered that could be used for rapid, inexpensive evaluation of phenylpropanoid and flavonoid characteristics related to seed meal quality or the yellow seed trait of elite *Brassica* germplasm. An HPLC-based phenolic fingerprint was identified that detected a pattern of phenolic acids consistent with the presence of the A genome in *Brassica* species.

Dihydroflavanol reductase (*DFR*) transcripts from developing whole seed were absent or less abundant at 5, 10, 20 and 30 days after pollination in a yellow-seeded line compared to a brown-seeded line of *B. carinata*. Dihydroflavonol reductase (*DFR*) transcripts in seedling leaf tissue from the yellow-seeded line grown at 20 to 25°C were less abundant compared to similarly grown brown-seeded material. Leaf tissue from seedlings grown at 15 to 18°C contained similar amounts of *DFR* transcript in both yellow- and brown-seeded lines of *B. carinata*. Data from these experiments suggest that seed coat pigmentation in the Brassicaceae is due to condensed tannin and phlobaphene accumulation, not anthocyanins, and that seed coat pigment biosynthesis is down-regulated at *DFR*, a structural gene in the flavonoid pathway. The regulatory factor controlling *DFR* expression in developing seed may have pleiotropic effects on anthocyanin biosynthesis in seedlings. In addition, there could be pleiotropic effects in related metabolic pathways. Thioglycolic lignin concentration was significantly lower in the yellow-seeded Brassicaceae and in the seed coat tissue of yellow-seeded *B. carinata* compared to dark-seeded samples.
Acknowledgements

"It was the best of times; it was the worst of times." (Dickens, In A Tale of Two Cities)

For their guidance in completing this research topic and in writing this dissertation, I sincerely thank my co-supervisors, Dr. Margaret Gruber and Dr. Graham Scoles. I am very grateful to Dr. Brian Ellis for serving as an excellent external examiner. In addition, I thank my advisory committee for their helpful comments regarding the various facets of my research: Dr. Peta Bonham-Smith for advice on RNA preparation, RT-PCR assays and Northern hybridizations; Dr. Alister Muir for advice on preparation and identification of plant natural products; Dr. Gerhard Rakow for advice on plant breeding and Brassica germplasm; Dr. Daryl Somers for his supervision of the molecular marker experiments and many helpful discussions; Dr. R. Tyler, who very generously joined my committee late in my program. As well, I thank Dr. Heather Ray, post-doctoral associate in Dr. Gruber’s lab, and Ms. Laureen Blahut-Beatty, technician, for invaluable help in designing primers, harvesting 5-dap seeds and troubleshooting RT-PCR assays and Mr. Andrew Urmanson, summer assistant, for help dissecting seed coat tissue and histochemical staining. In addition, I am very grateful to Mr. Jim Elder and Ms. Krista Thompson for assistance with analyses by LC-MS and Mr. Ralph Underwood for his extensive expertise in converting the photographs and figures to computer-based graphics suitable for my dissertation.

Most importantly, I thank my husband, Eric Marles, for his tremendous personal encouragement. I would not have survived without his willingness to share his life with the chaos a doctoral dissertation brings. For the all my friends who helped make it the spring of hope, when it seemed like the winter of despair, I thank you for your steadfastness. In addition, I am extremely grateful for scholarships from NSERC and the University of Saskatchewan. I also thank Agriculture & Agri-Food Canada for support when my scholarships finished.
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List of Abbreviations

\( \lambda \) wavelength

\( \mu E \) microEinstein (measure of photosynthetically active radiation; units, \( \mu \text{mol m}^{-2} \text{s}^{-1} \))

\( \mu m \) micrometre, micron (1 x 10\(^{-6}\) metre)

\( ^{\circ} \text{C} \) degrees Celsius

1D, 2D one dimensional, two dimensional

4CL 4-coumarate:coenzyme A ligase

AAFC Agriculture and Agri-Food Canada, Saskatoon, Canada

AC Agriculture Canada (prefixed to an AAFC-registered cultivar)

APcI atmospheric pressure chemical ionization

AS anthocyanin synthase

ast \( \text{Arabidopsis spotted testa mutant} \)

AU absorbance units

BAN \( \text{BANYULS (ban, Arabidopsis anthocyanin seed coat mutant)} \)

BAW \( n\)-butanol : HOAc : water (4:1:5) (TLC solvent)

bHLH basic helix-loop-helix amino acid motif

bp base pair

BSA bulked segregant analysis

BuOH-HCl \( n\)-butanol-hydrochloric acid

bZIP basic leucine zipper

cDNA complementary DNA

C4H cinnamate 4-hydroxylase

c a circa

CAW CHCl\(_3\) : HOAc : water (30:15:2) (TLC solvent)

CHCl\(_3\) chloroform

CHI chalcone isomerase (protein); \( CHI \) (gene)

CHS chalcone synthase (protein); \( CHS \) (gene)

cm centimeter

cM centi-Morgan; a genetic map unit

CoA co-enzyme A
conc. concentrated
cpm counts per minute
CT condensed tannin
CTAB cetyl trimethylammonium bromide
cv cultivar, cultivated variety
d days
da dalton
dap days after pollination
DEPC diethylpyrocarbonate
DFR dihydroflavonol reductase (protein); DFR (gene)
DH doubled-haploid
DHK dihydrokaempferol
DHM dihydromyricetin
DHQ dihydroquercetin
DNA deoxyribonucleic acid
EDTA ethylenediamine disodium tetra-acetate
e.g. for example (exempli gratia)
ER endoplasmic reticulum
etc. and others (et cetera)
EtBr ethidium bromide
EtOAc ethyl acetate
EtOH ethanol
F filial
F3H flavonol-3-hydroxylase (protein); F3H (gene)
F3'H flavonol-3'-hydroxylase (protein); F3'H (gene)
F3'5'H flavonol-3'5'-hydroxylase (protein); F3'5'H (gene)
kb kilobase (1000 bp)
f.g. field-grown
g gram
g gravitational force
h hour
HOAc  acetic acid
HPLC  high performance liquid chromatography
i.i.  inner integument
i.e.  that is (ille)
IR  infra-red
Kb  kilobase
LCMS  liquid chromatography-mass spectrometry
LDOX  leucoanthocyanidin dioxygenase
m  meter
M  molar
mAU  milli-absorbance units
MeOH-HCl  methanol-hydrochloric acid
min  minute
MOPS  3-N-morpholino propanesulphonic acid
mRNA  messenger RNA
MS  mass spectrometer
NCBI  National Centre for Biotechnology Information
n.d.  not detected
N.D.F.  neutral detergent soluble fibre
ng  nanogram
NI  negative ion mode
nm  nanometer
NMR  nuclear magnetic resonance
No.  number
N.S.P.  detergent soluble non-starch polysaccharides
nt  nucleotide
o.i.  outer integument
PAL  phenylalanine ammonia lyase (protein); PAL (gene)
pal.  palisade
PAR  photosynthetically active radiation
PBI  Plant Biotechnology Institute, Saskatoon, Canada
<table>
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<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PDA</td>
<td>photodiode array</td>
</tr>
<tr>
<td>PI</td>
<td>positive ion mode</td>
</tr>
<tr>
<td>PTFE</td>
<td>teflon-lined (literally, ‘PolyTetraFluoroEthylene’)</td>
</tr>
<tr>
<td>PVPP</td>
<td>polyvinylpolypyrrolidone chloride</td>
</tr>
<tr>
<td>R&lt;sub&gt;f&lt;/sub&gt;</td>
<td>ratio to front (relative mobility); usually multiplied by 100</td>
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<tr>
<td>RFLP</td>
<td>restriction fragment length polymorphism</td>
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<td>RNA</td>
<td>ribonucleic acid</td>
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<td>RP</td>
<td>reversed-phase</td>
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<td>RT</td>
<td>retention time</td>
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<td>second</td>
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<td>S</td>
<td>selfed</td>
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<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SE</td>
<td>standard error of the means</td>
</tr>
<tr>
<td>SETS</td>
<td>Salt, EDTA, Tris-Sodium pyrophosphate buffer</td>
</tr>
<tr>
<td>SIR</td>
<td>single ion recording</td>
</tr>
<tr>
<td>SRS</td>
<td>Saskatoon Research Centre, AAFC</td>
</tr>
<tr>
<td>SCC</td>
<td>saline sodium citrate buffer</td>
</tr>
<tr>
<td>TAE</td>
<td>tris-acetic acid, EDTA buffer</td>
</tr>
<tr>
<td>TIC</td>
<td>total ion current</td>
</tr>
<tr>
<td>TLC</td>
<td>thin layer chromatography</td>
</tr>
<tr>
<td>t&lt;sub&gt;t&lt;/sub&gt;</td>
<td>transparent testa (Arabidopsis seed coat mutants)</td>
</tr>
<tr>
<td>TFA</td>
<td>trifluoroacetic acid</td>
</tr>
<tr>
<td>UBC</td>
<td>University of British Columbia</td>
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<tr>
<td>UV</td>
<td>ultraviolet</td>
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<td>V</td>
<td>volt</td>
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<td>ver.</td>
<td>version</td>
</tr>
<tr>
<td>VHO</td>
<td>very high output</td>
</tr>
<tr>
<td>v/v</td>
<td>volume to volume</td>
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<tr>
<td>WT</td>
<td>wild type</td>
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<tr>
<td>w/v</td>
<td>weight to volume</td>
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DEDICATION

This dissertation is dedicated to the memory of my Mother, Helen Alberta Swain-Willis, who always encouraged me to acquire as much education as I could; and to my daughters, Shannon Jaleen and Allison Patricia, who both inspired and encouraged me as students, ‘we three together’.
CHAPTER 1

INTRODUCTION

Species of *Brassica* are grown worldwide to produce seed for either an edible oil (*Brassica napus* L., *B. rapa* L.) or mustard (*B. juncea* L. Czern., *B. carinata* A. Braun; ‘Ethiopian mustard’). In the prairie region of Canada, 8.63 million tonnes of *B. napus* and *B. rapa* were produced in 1999 to provide seed for canola oil (FAO Statistics on World Crops, 1999). Currently, canola meal is undervalued compared to soybean meal for supplementing animal feed and in the food industry. This is due, in part, to anti-nutritional, polyphenolic substances such as condensed tannins (proanthocyanidins) and indigestible, fibre-related compounds (*e.g.* lignin) (Bell and Shires, 1982; Bell, 1993). With modifications to the phenolic profile of *B. napus*, canola meal would be more useful as a livestock feed or as a gluten-free supplement in human nutrition (Statistics Branch, Saskatchewan Agriculture and Food, 1998).

1.1 The importance of the unpigmented seed coat trait

For over twenty years, plant breeders have attempted to develop yellow-seeded canola cultivars as a way of improving meal quality and increasing oil concentration in *Brassica* species (Fowler and Downey, 1970; Stringam *et al.*, 1974; Jönsson, 1977; Stringam, 1980; Hawk, 1982; Poulsen *et al.*, 1991). The ‘yellow seed’ characteristic is actually a seed with an unpigmented testa or seed coat. The ‘yellow’ appearance is due to the colour of the embryonic cotyledons which are visible through the nearly colourless layers forming the seed coat (Bouman, 1975; Buth and Ara, 1981). Seed
meal analyses have shown that phenolics and fibre content are lower in yellow-seeded cultivars of *Brassica* species than in dark-seeded cultivars (Bell, 1993; Simbaya *et al.*, 1995). *Brassica carinata* could be useful for improving canola cultivars in Canada due to its dominant trait for the 'yellow seed' character. *Brassica carinata* differs from *B. napus* because yellow seed colour is apparently controlled by one gene that is partially dominant over the black or brown seed colour (Getinet and Rakow, 1997), whereas in *B. napus*, the yellow seed colour is controlled by at least three recessive loci (Shirzadegan, 1986; Hou-Li *et al.*, 1991; Van Deynze and Pauls, 1994). In addition, *B. carinata* has desirable agronomic traits (large seeds, heat-, drought- and disease-tolerance, less shattering compared to cultivars of *B. napus* or *B. rapa*) and such traits are advantageous for successful adaptation to the Canadian prairies (Gugel *et al.*, 1990; Getinet *et al.*, 1996; Rakow and Getinet, 1998; Falk, 1999).

However, yellow-seeded *B. napus* has proven difficult to develop due to the recessive nature of the multiple genes associated with the unpigmented seed coat trait (Yousuf, 1982; Shirzadegan, 1986; Wang and Liu, 1991). One plant breeding strategy depended on interspecific crosses between *B. carinata* and *B. napus* to produce yellow-seeded *B. napus* cultivars by incorporation of the gene controlling the unpigmented seed coat trait of *B. carinata* (Rashid *et al.*, 1994). This approach was not entirely successful because the *B. napus* progeny from such crosses produced seed that was heavily mottled. Canola breeders may be able to develop the elusive "all-yellow seed" cultivar of *B. napus* by better understanding how the genetically regulated repression of seed coat pigmentation functions in *B. carinata*.
1.2 DNA-based insights into seed coat pigmentation

A genomic analysis approach was used in soybean (*Glycine max* L.) to elucidate genetic control of seed coat pigmentation. Sequencing of restriction fragment length polymorphisms (RFLPs) associated with seed coat pigmentation showed that these DNA sequences were related to chalcone synthase (CHS), the first enzyme in the flavonoid pathway (Akada and Dubc, 1995; Todd and Vodkin, 1996). In a study of *B. napus*, CHS gene sequences were also discovered to be linked to the seed coat pigmentation trait when RFLPs in DNA of doubled haploid populations were identified by Van Deynze et al. (1995). Thus, the development of a completely yellow-seeded *B. napus* cultivar may be assisted by using DNA sequence information related to enzymes of the flavonoid pathway to find RFLPs associated with DNA linked to the seed coat pigmentation trait. Such RFLPs would be used to screen DNA from progeny of interspecific crosses such as *B. carinata* x *B. napus* to guide selection of new *Brassica* germplasm.

Flavonoid gene expression patterns in mutants have helped to explain genetic control of seed coat pigmentation. The lack of visible seed pigmentation in transparent testa mutants (*tt* mutants) of *Arabidopsis thaliana* L. Heynh. Has been correlated with lower transcript levels, altered transcript or entirely missing transcripts of genes in the phenylpropanoid/flavonoid pathways (Shirley et al., 1995). However, *Arabidopsis* has a small genome (1.3 x 10^6 base pairs [bp]) (Arabidopsis Genome Initiative, 2000) and in many cases, a given trait is controlled by a single copy of a gene. If the single copy of a gene affecting the production of pigments is mutated or regulated differently, pigment production may be affected in all plant tissues, including the seed coat of the mutant (Shirley et al., 1995).
By comparison, *B. carinata* originated from the two ancestral diploid genomes of *B. oleracea* L. and *B. nigra* L. Koch. (U, 1935; 'Triangle of U', Appendix A). Even in diploid species, there are many traits controlled by multiple copies of a given gene, e.g. chalcone synthase (CHS) in soybean (Todd and Vodkin, 1996). Although it could be complicated to find the gene responsible for the control of pigmentation in seed coats in an amphidiploid species like *B. carinata*, a candidate gene or gene family could be inferred from differences in the phenylpropanoid and flavonoid profiles present in the seed coats of yellow- versus brown-seeded plants. Identification of the phenolic compounds in the seed coats from closely-related lines of yellow- and brown-seeded *B. carinata* may indicate which biosynthetic precursors for pigment compounds are absent or have accumulated in the yellow seed lines. This 'phenolic signpost', combined with a gene expression study, may identify the gene(s) responsible for the differences in seed coat pigmentation.

Researchers have shown a positive correlation between lower fibre content and the lack of seed coat pigmentation in *Brassica* spp. (Mitaru *et al.*, 1982; Shirzadegan and Röbbelen, 1985; Getinet, 1986; Simbaya *et al.*, 1995). Specific lignin determination, rather than the traditional measurement of crude or total dietary fibre may identify whether this phenolic polymer is significantly reduced in yellow-seeded *Brassica* species compared to brown-seeded species.

1.3 Research objectives

The objectives of this research were (1) to identify the chemical characteristics of seed coat pigment in selected Brassicaceae by histochemical staining and seed extraction assays. This analysis was expected to provide an overview of the chemical classification of the pigments and a record of pigmentation patterns in the germplasm.
of agronomically-important Brassicaceae relative to *B. carinata*. (2) to identify
differences in the phenylpropanoid and flavonoid profiles of genetically-related lines of
yellow- and brown-seeded *B. carinata*. This was expected to indicate which of the
phenylpropanoid or flavonoid structural genes is most likely linked to the yellow-seed
trait. (3) to identify RFLPs between genetically-related yellow- and brown-seeded *B.
carinata*, using probes derived from seed pigmentation-related loci, *e.g.* *CHS* and (4) to
document the expression of a candidate structural gene involved in pigment synthesis
in developing seed coats, based on variation in the phenylpropanoid/flavonoid chemical
profiles determined according to the second objective.
CHAPTER 2

LITERATURE REVIEW

2.1 Seed coat tissue development in Brassica species

Seed coat material is parental tissue derived from the integuments of the ovule of the maternal plant. The development of the integuments is the first morphological change to occur in the ovule primordium (located in the floral meristem) in Arabidopsis (Gasser et al., 1998). The primordium differentiates into three layers before the nucellus (the structure preceding development of the embryo sac) is fully formed (Angenent and Colombo, 1996). The inner and outer integuments and the cells that ultimately form the columnar palisade layer in the outer integument are derived from this early three-layer differentiation (Bouman, 1975; Corner, 1976; Van Caeseele et al., 1982). In Arabidopsis, the formation of each layer is under independent genetic control (Gaiser et al., 1995; Klucher, 1996).

2.1.1 Localization of pigment within the developing seed coat

In Brassica species, pigments are deposited on the inner side of the seed coat, next to the palisade layer in the cells forming the inner integument (i.i.) (Corner, 1976; Van Caeseele et al., 1982) (Figure 2.1, A). Cytological staining has confirmed that the inner integument in young seeds of B. napus contain phenolics that are concentrated against the palisade layer as the embryo develops (Iwanowska et al., 1994). Seed coat palisade cells are crushed against the outer epidermis during embryo development and form a layer that is rigid and persistent (Corner, 1976).
Figure 2.1. Cross-section of the seed coat in *Brassica* sp.

(A) Developing seed coat, with intact outer epidermis, outer integument (o.i.), inner integument (i.i.). Pigment deposits scattered throughout cytoplasm of the i.i (1) and in a layer (2) adjacent to the columnar, thick-walled palisade layer.

(B) Mature seed coat after compression of i.i and pigments against the secondary thickening of palisade layer. The depleted endosperm (endo.) and i.i. cytoplasm have disintegrated and embryonic cells (3) are now pressed against the remains of i.i. and the pigment layer (dashed lines joining A to B). Intercellular space (4) arises from the disintegration of the outer epidermis. Eventually the outer epidermis sloughs off and a lignified, ridged layer of palisade cells remains.

(Adapted from Corner, 1976).
Large empty areas, devoid of cytoplasm, remain next to the outer epidermis, preserved by the rigidity of the palisade layer. In fully matured *B. napus* seeds, cellular debris has sloughed off and the outer integument consists only of palisade cells (Iwanowska *et al.*, 1994). The maturing embryo compresses the inner integument and the contents of the inner integument (including the pigment) are deposited on the inner side of the palisade layer (Figure 2.1, B). The palisade cells in matured seed coats in *Brassica* species are fully developed, thicker-walled cells compared to other members of the Tribe Brassiceae (Family Brassicaceae) (Bouman, 1975; Corner, 1976; Gómez-Campo, 1980). Unlike seeds in monocots, endosperm material is depleted in *B. napus* as the cotyledons develop and the endosperm disappears by the time the embryo is a month old (Fowler and Downey, 1970). The pigments are concentrated and subject to oxidation as the seed matures and loses water (Bergfeld and Schopfer, 1986). The palisade cells are strengthened by secondary thickening and form the protective, outermost layer in the matured seed (Bouman, 1975). Germination of the mature embryo does not involve the extruded pigments or seed coat tissue (Gómez-Campo, 1980; Buth and Ara, 1981; Van Caeseele *et al.*, 1982; Bergfeld and Schopfer, 1986; Iwanowska *et al.*, 1994).

### 2.2 Phenylpropanoid and flavonoid biosynthesis

Compounds that contribute to pigment synthesis are derived from phenylpropanoids (Figure 2.2, structures in blue) and flavonoids (Figure 2.2, structures in black). Phenylpropanoids are produced from the aromatic amino acid, phenylalanine and to a lesser extent, tyrosine in some plant species (Herrmann, 1995; Douglas, 1996). Malonyl-CoA (an acetate metabolite) and *p*-coumaryl-CoA are combined by chalcone synthase to form chalcones (Figure 2.2, structure in red). The chalcone molecules are
Figure 2.2 A schematic diagram of the phenylpropanoid (blue structures), and flavonoid (black structures) pathways. (Adapted from Shirley [1996]; Styles and Ceska [1989]).

Chalcone (red structure) is formed from three molecules of malonyl-CoA and one molecule of \( p \)-coumaric acid. ‘R’ represents positions which may be substituted by hydroxyl-, methoxy- or glycosyl- groups. Numbers and acronyms adjacent to arrows indicate enzymes as follows:

1. Phenylalanine ammonia lyase (E.C. 4.3.1.5; PAL)
2. Cinnamate-4-hydroxylase (E.C. 1.14.13.11; C4H)
3. 4-coumarate:coenzyme A ligase (E.C. 6.2.1.12; 4CL)
4. Chalcone synthase (E.C. 2.3.1.74; CHS)
5. Chalcone isomerase (CHI)
6. Flavanone-3-hydroxylase (F3H)
7. Dihydroflavonol reductase (DFR)
8. Flavonol synthase (FS)
9. Leucoanthocyanidin reductase (LAR)
10. Anthocyanin synthase (AS) (or ‘LDOX’; leucoanthocyanidin dioxygenase)
11. 3-O-glycosyltransferases

*end product synthesis illustrated in more detail. Figure 2.4.
converted to flavanone compounds, the first intermediate in the flavonoid pathway; thus ring A of this flavonoid molecule is a polyketide (*i.e.* acetate in origin via the three malonyl-CoA molecules) (Figure 2.3, black subdivision of the molecule). Ring B originates from phenylalanine by way of p-coumaryl-CoA (Figure 2.3, red segment of the molecule) and these together complete Ring C (Mann, 1987).

### 2.2.1 Effect of enzyme substrate accumulation on flavonoid metabolism

The accumulation of enzyme substrates can affect the enzymatic activity in both the catalytic step immediately previous (feed-back control) or in synthetic steps beyond the precursor (feed-forward control). In experiments using a range of *t*-cinnamic acid concentration (0.02 to 1 mM), measurement of *in vitro* PAL activity extracted from pea (*Pisum sativum* L.) showed that PAL activity was absent at the higher levels of *t*-cinnamic acid concentration and partially inhibited at the lowest concentrations (Shields *et al.*, 1982). Subsequently, *t*-cinnamic acid was shown to down-regulate PAL activity in legumes and flavonoid biosynthesis was affected (Bolwell *et al.*, 1986, 1988). Suspension cultures of common bean (*P. vulgaris*), treated with 1.0 mM exogenous *t*-cinnamic acid, were used to measure the effect on PAL enzyme activity and protein degradation (Bolwell *et al.* 1986, 1988). Addition of 1.0 mM *t*-cinnamic acid resulted in the loss of PAL activity, arrested CHS enzyme activity and depressed the rate of translation of *PAL* and *CHS* mRNAs. Hrazdina and Jensen (1992) and Hrazdina (1992) have suggested that these experiments demonstrating feedback inhibition for controlling PAL activity were conducted with non-physiological concentrations of *t*-cinnamic acid and that the suspension cell cultures used did not behave like *in planta* systems with intact metabolic pathways, regulation and tissue
Figure 2.3 The biosynthetic origins of a flavonoid molecule.

The formation of a flavonoid requires the incorporation of deaminated phenylalanine (a product of the shikimate pathway [in red]) and three molecules of malonyl-CoA (synthesized by acetyl-CoA carboxylase) which combine to form the acetate portion (structure in black). The carbons are numbered according to whether they occur in the A and C ring (unprimed numbering) or the B ring (primed numbering). (Adapted from Mann, 1987)
differentiation. However, nonspecific enzyme inhibition by the exogenous t-cinnamic acid was unlikely because nine other polypeptides were induced by t-cinnamic acid treatment of the bean cultures (Bolwell et al., 1988). In addition, the presence of 0.1 mM t-cinnamic acid in elicitor-induced, bean cell suspension-cultures selectively affected the appearance of new PAL mRNA without impairing transcription of the constitutively produced transcripts or affecting the appearance of transcripts for the elicitor-induced enzymes, glucanase and chitinase (Mavandad et al., 1990). These authors also found that exogenous t-cinnamic acid can behave as a negative regulator of PAL, since constitutive enzyme activities and other flavonoid transcripts (e.g. CHI) were not affected and additional polypeptides were induced.

The accumulation of dihydroflavonols and flavonols can be correlated with the lack of an endproduct in the pathway (anthocyanin). In experiments with a series of petunia (Petunia hybrida Hort. ex Vilm.) mutants defective in anthocyanin formation in the flowers, the metabolic fate of specific dihydroflavonols and flavonols was compared to the wild type (WT) petunia (Gerats et al., 1982; Forkmann and Ruhnau, 1987; Beld et al., 1989). Substrate specificity and final products synthesized from dihydroflavonols in petunia mutants showed that specific anthocyanins and flavonols are formed as end products and that the pathway is highly mutable (Gerats et al., 1982; Forkmann and Ruhnau, 1987). Flavonoid 3'-hydroxylase (F3'H) and flavonoid 3'5'-hydroxylase (F3'5'H) are enzymes responsible for adding the hydroxyl group(s) to the B ring of flavonoid substrates, naringenin (a flavanone) or dihydrokaempferol (Stotz et al., 1985; Forkmann and Ruhnau, 1987). In WT petunia floral tissues, dihydrokaempferol is preferentially converted by F3'5'H to dihydromyricetin, a DFR substrate and ultimately forms an anthocyanin, delphinidin (Forkmann and Ruhnau,
Dihydrokaempferol was not converted to an anthocyanin (hence there was no pelargonidin) and was found to accumulate in minimal amounts along with dihydroquercetin (Gerats et al., 1982).

In one mutant, there were normal DFR, F3’H and FS activities but no F3’5’H activity so dihydromyricetin was not produced and kaempferol and quercetin were preferentially accumulated, rather than their respective anthocyanins (Gerats, 1985, cited in Holton and Cornish, 1995). In petunia lines that exhibited F3’H activity and lacked both F3’5’H and FS activities, cyanidin (an anthocyanin) rather than quercetin was produced, even though dihydroquercetin was normally a poor substrate in petunia for DFR (Beld et al., 1989; Forkmann and Ruhnau, 1987). Beld et al. (1989) analyzed another mutant to show that dihydrokaempferol always proceeded to delphinidin (not cyanidin) in the presence of both F3’H and F3’5’H activities when FS activity was absent. Dihydroquercetin, formed by F3’H from dihydrokaempferol, was converted to dihydromyricetin by F3’5’H and thence to delphinidin rather than synthesizing cyanidin from dihydroquercetin.

The final mutant in this group did not form any anthocyanins although all enzyme activities were reported to be present (DFR, F3’H, F3’5’H, FS). The flavonols kaempferol and quercetin accumulated and there was no myricetin even though F3’5’H activity was present to form dihydromyricetin (Beld et al., 1989). It was proposed that, due to competition between these three enzymes and DFR, the most catalytically efficient enzyme might be converting the common substrate, dihydrokaempferol, into flavonol end-products. Although Beld et al., (1989) do not discuss catalytic constants or explain exactly how substrate specificities were determined in each set of mutants, Stafford (1990, 1991) proposed that these flavonoid enzymes might be organized into a
multienzyme complex that contributed to substrate channelling and affected the availability of the dihydroflavonols to DFR compared to FS. Holton and Cornish (1995) speculated that regulatory genes play a large part in the expression of the enzymes that utilize common substrate pools such as dihydrokaempferol. These authors concluded that petunia regulatory genes control expression of essentially all the anthocyanin-related structural genes after F3H (the enzyme that converts the flavanone, naringenin to dihydrokaempferol).

2.3 Phlobaphenes, condensed tannins and anthocyanins in seed coats

Visible flavonoid pigmentation in seed coats is generally due to three classes of compounds: phlobaphenes, condensed tannins and anthocyanins. Condensed tannins are usually colourless in immature tissue (Harborne, 1967; Harborne et al., 1975) as are flavan-4-ols, precursors of phlobaphenes (Styles and Ceska, 1975, 1977). These polymers become visibly brown or black in seed coats of sorghum (Sorghum bicolor L.), soybeans and Sinapis alba L. from the effects of oxidation and tissue dehydration during seed maturation (Bernard and Weiss, 1973; Bergfeld and Schopfer, 1986; Stafford, 1990). Anthocyanins can be so deeply coloured in mature soybean seed coat that the seed appears black (Buzzell et al., 1987). Black bean (Phaseolus vulgaris L) contains high concentrations of delphinidin, petunidin and malvidin 3-O-glucosides, anthocyanins that are easily extracted by acidified methanol from mature, isolated seed coat tissue (Takeoka et al., 1997).

Yellow and beige colours have also been reported in seed coat pigmentation in legumes. For example, kaempferol glycosides were the only flavonoids found in the dry bean cultivar 'Prim' (P. vulgaris) and these flavonols were considered responsible for imparting a yellow colour to the mature seed coat (Beninger and Hosfield, 1998).
2.3.1 Phlobaphenes and condensed tannins

Phlobaphenes are brown pigments produced from flavanones through a flavan-4-ol intermediate (Figure 2.2) (Hahlbrock and Grisebach, 1979; Styles and Ceska, 1989). From the earliest reports, in vitro condensation and polymerization reactions involving flavanones or flavonols produced brown pigments chemically similar to phlobaphenes of maize (*Zea mays L.*) pericarp and legume seed coat (Stähelin & Hofstetter, 1844, cited in Nagai, 1921).

More recently, unextractable red-brown pigments from maize pericarps and cobs were classed as phlobaphenes and a biosynthetic pathway incorporating flavan-4-ols was established (Styles and Ceska, 1975, 1977). Currently, there is no specific assay for phlobaphene pigments, and they are identified on the basis of an unchangeable, brown or red-brown colour after negative assay results for anthocyanins and condensed tannins (Harborne, 1997). Phlobaphenes do not undergo acidic hydrolysis to form characterizable monomers (Harborne, 1984). In the presence of hot *n*-butanol-HCl (BuOH-HCl), phlobaphenes do not form the characteristic red compound that is a positive test for condensed tannins (Watterson and Butler, 1983).

Condensed tannins are produced by the condensation of flavan-3-ol units and flavan-3,4-diols (Figure 2.4). Flavan-3,4-diols (*e.g.* leucoanthocyanidins) are usually transient molecules in the metabolic pool and polymerize via a carbocation (positively-charged carbon on the flavonoid A ring) to form a linear (or branched) polymer with a molecular mass ranging from 500 to more than 20,000 daltons (da) (Swain, 1979; Stafford, 1983; Kristiansen, 1986). Dihydroflavonol reductase uses the dihydroflavonols to synthesize flavan-3,4-diols and flavan-4-ols (Stafford, 1983; Froemel *et al.*, 1985).
4,8-linked polymer of condensed tannin

Figure 2.4  Formation of condensed tannins and anthocyanins from dihydroflavonol.

Enzymes are numbered as follows (1) DFR, (2) LAR, (3) AS (LDOX), (4) Anthocyanin glycosyl transferase. R = -H, -OH, or -O-glycoside.

Adapted from Styles and Ceska (1977) and Stafford (1983).
Leucoanthocyanidin reductase (LAR) catalyzes the step converting colourless flavan-3,4-diols to flavan-3-ols (e.g. catechin and gallicatechin), enroute to formation of condensed tannin oligomers (Jende-Strid, 1991, 1993). The oligomers polymerize to form a colourless product which dehydrates and oxidizes to form a brown to black pigment. This becomes visible in the mature seed coat as brown to black pigments (Stafford, 1990). The details of the final condensing steps are not known yet, but the reactions appear to be mediated by enzymes (Jende-Strid, 1993).

The flavonoid content of the seeds of *A. thaliana* in the Family Brassicaceae, was determined both in the wild-type and *tt* mutants (Kubasek *et al.*, 1992; Sheahan and Rechnitz, 1993; Shirley *et al.*, 1995; Shirley, 1996; Kubasek *et al.*, 1998). Pigmentation in seed coats from dark brown-seeded wild-type and light brown-seeded *A. thaliana* mutants *tt*6 and *tt*10 (Sheahan and Rechnitz, 1993; Shirley *et al.*, 1995: Shirley, 1996) was attributed to condensed tannins and co-pigmentation compounds (flavones and flavonols). In oilseed cultivars of flax (*Linum usitatissimum* L.), dark-seeded cultivars were found to contain more phenolics than yellow-seeded lines, and the visible pigments were identified as condensed tannins (Oomah *et al.*, 1995).

Seed coat-related condensed tannins have been reported in the pericarp and testa of maize, barley (*Hordeum vulgare* L.) and sorghum (Price and Butler, 1977; Aastrup *et al.*, 1984; Styles and Ceska, 1989). Pericarp tissue in maize, barley and sorghum is derived from the ovary wall (Esau, 1964). The true seed coat (the layers covering the ovule) is fused to the pericarp; consequently, seed coat- and pericarp-based pigments are difficult to distinguish separately in the matured caryopsis (kernel) (Sanders, 1955). Peeled caryopses were used initially in condensed tannin determinations in barley, but the layers were contaminated either with underlying aleurone material or the outer
pericarp, so the exact location of the condensed tannins was unknown (Jende-Strid, 1978). This difficulty was resolved by specifically staining for condensed tannins in sectioned half-seeds (Aastrup et al., 1984). Based on this histological technique, condensed tannins in barley and sorghum are located only in the testa layer.

Seed coat pigment deposition in barley and sorghum is similar to that in developing ovules and seed coats in the Brassicaceae and is genetically regulated separately from other tissues (Jende-Strid, 1993), comparable to reports for Arabidopsis (Gaiser et al., 1995; Klucher, 1996; Gasser et al., 1998). The caryopses of barley and sorghum mature before the pigments in the testa layers became visible (Sanders, 1955; Aastrup et al., 1984); however, some pigmentation is visible in early stages of developing tissues in maize pericarp material (Styles and Ceska, 1989).

Condensed tannins and anthocyanins in seed, leaf and floral tissues from legume species can be distinguished from one another based on their reaction to histochemical stains (Sarkar and Howarth, 1976; Lees et al., 1993; Lees et al., 1995; Skadhauge et al., 1997). In the forage legume, sainfoin (Onobrychis viciifolia Scop.), fresh plant tissue was examined by using two reagents in sequence: ethanol (EtOH) acidified with conc. HCl (2:1, v/v) and 10% vanillin acidified with HCl (Lees et al., 1993). When anthocyanins were present, red colour appeared in the tissue within 2 min of the addition of the acidified alcohol solution. This staining method discriminated between anthocyanins and the presence of condensed tannins because condensed tannins did not stain as rapidly (Lees et al., 1993). Due to the slow cleavage (hydrolysis) of the polymer, condensed tannins produced a bright cherry red colour in the presence of the vanillin-HCl after >30 min.
Differential staining to distinguish anthocyanins and condensed tannins was used to determine the presence or absence of condensed tannins in alfalfa (*Medicago sativa* L.) seeds (Skadhauge *et al.*, 1997). A brown-red colour developed slowly in alfalfa seed coat sections treated with BuOH-HCl and there was no instantaneous red stain upon the addition of either the BuOH-HCl or 50% HCl. These observations were consistent with the data presented by Lees *et al.* (1993) where condensed tannins hydrolyzed slowly to form red products (anthocyanidins) when the material was acidified whereas anthocyanins turned red immediately (<2 min). The selectivity of reagents used to determine condensed tannins is discussed further in Appendix B.1.

### 2.3.2 Anthocyanins in seed coats

Most of the red and blue pigments in plants, as well as variations from pink and orange to violet hues, belong to the anthocyanin family of flavonoids (Harborne *et al.*, 1975; Jackman *et al.*, 1987). Anthocyanins are produced from the same pool of leucoanthocyanidin precursors used to form condensed tannins (Figures 2.2, 2.4). Anthocyanin synthase mediates ‘anthocyanidin’ formation (characterized by a charged C-ring) and is subject to regulation through complex signal transduction networks (reviewed by Mol *et al.*, 1996). Several enzymes modify the anthocyanidin aglycone by substituting glycosidic groups (*e.g.* monosaccharaides) on the A- and C-rings and the C-3’, C-4’ and/or C-5’ positions on the B-ring (Harborne, 1967; Harborne and Williams, 1995) (Figure 2.4). These pigments are colourless except in an acidic environment such as the vacuole (Harborne and Williams, 1995). Compared to condensed tannins, anthocyanins are more easily assayed because they can be readily extracted from plant tissue and separated by thin-layer chromatography (TLC).
Anthocyanins have not been conclusively identified in seed coat tissue of the Brassicaceae species. The presence of ‘anthocyanidins’ was reported in Arabidopsis seeds (Sheahan and Rechnitz, 1993; Shirley et al., 1995; Albert et al., 1997; Devic et al., 1999), but the anthocyanidin molecule could have originated from hydrolyzed condensed tannins rather than anthocyanins, due to the acidic nature of the tissue preparation in these studies. Otherwise, the Brassicaceae readily produce anthocyanins in leaves, stems and siliques (Hodges and Nozzolillo, 1996). Occasionally, anthocyanins accumulate in a species-specific manner in anthocyanoplasts, specialized vesicles formed from the tonoplast in leaves (Nozzolillo and Ishikura, 1988). Brassica oleracea leaf tissue has been reported to accumulate anthocyanins in anthocyanoplasts (Hodges and Nozzolillo, 1996); however, anthocyanins in B. napus cv Westar seedlings accumulated throughout the vacuole, turning that organelle red, and anthocyanoplasts were not observed (Nozzolillo and Ishikura, 1988; Hodges and Nozzolillo, 1996).

Anthocyanins are the most common pigments in legume seed coats, where delphinidin, malvidin, petunidin, pelargonidin and paeonidin create shades ranging from blue through purple, red and orange colours (Harborne et al., 1975). In seed coats from violet-coloured broad bean (Vicia faba L.), the pigment was identified as an anthocyanin (Nozzolillo and Ricciardi, 1992). These compounds have been detected in cultivars of bean (P. vulgaris and Vigna spp.), lentil (Lens spp.) and soybean (G. max) that have red, purple, brown or black pigmented seed coats (Buzzell et al., 1987; Nozzolillo and Ricciardi, 1992; Onyilagha, 1994; Yoshida et al., 1996).

2.4 Genetic regulation of pigmentation in seed coat and pericarp

Research into the control of seed coat pigmentation in the Brassicaceae is still in its infancy compared to the body of literature and the resources that have been devoted
to legume and maize research. As a result of the studies that identified the chemical basis of seed coat pigments and with the development of legume and maize breeding lines, a complex set of regulatory genes has been discovered that affects the pigmentation of the seed coat or pericarp.

2.4.1 Genetic regulation of seed colour in legumes

The genetics of seed coat colour in legumes is based on complex interactions among eight principal multi-allelic genes (Wang et al., 1994; Bassett, 1995; Beninger and Hosfield, 1999, 2000). The ground factor gene, \( P \), is required for synthesis of any pigment, otherwise the seed coat is colourless (\( p \)) or gray (\( p^{fr} \)). The genes \( C \) or \( J \) are needed to fully express the colour modifying alleles (\( G \) [yellow brown], \( B \) [green-brown], \( V \) [violet to black], \( r k \) [red]) and the presence of the locus, \( I \), can repress pigmentation. The homozygous, recessive form of the \( I \) allele, \( i \), specifies a fully pigmented seed coat, whereas, the genetic interaction, \( i^{k} \), inhibits the expression of pigment except at the hilum, and \( i^{k} \) restricts pigment to the saddle region of the soybean seed coat (Bernard and Weiss, 1973). The dominant red colour gene \( R \) is closely linked to \( C \) and is responsible for anthocyanin accumulation (Bassett, 1995), unlike \( r k \) which is a recessive allele responsible for condensed tannin synthesis in place of anthocyanins in certain cultivars of red kidney bean seed coat (Beninger and Hosfield, 1999, 2000).

2.4.2 Genetic regulation of pigmentation in maize and other genera

Maize, snapdragon (Antirrhinum majus L.) and petunia have also been used extensively to study genetic regulation of the flavonoid pathway and the production of pigments in the pericarp or floral organs (Styles and Ceska, 1975, 1989; Goff et al., 1992; Quattrocchio et al., 1993; Grotewold et al., 1994). In maize, it has been shown
that a regulatory protein, encoded by the $P$ gene, binds to and activates transcription of the $Al$ ($DFR$) gene required for both flavan-4-ol and flavan-3,4-diol biosynthesis (precursors for phlobaphenes, condensed tannins and anthocyanins) in pericarp, aleurone and floral organs (Styles and Ceska, 1975, 1977; Dooner et al., 1991; Tuerck and Fromm., 1994; Grotewold et al., 1994). Grotewold et al. (1994) determined that the MYB-homologous $P$ gene is a transcription factor that controls phlobaphene pigmentation in maize. In the presence of $P$, the protein encoded by $CI$ binds to the $DFR$ promoter to activate phlobaphene biosynthesis (Sainz et al., 1997). Other anthocyanin regulatory genes ($R/B$) were also found to affect the pigmentation in specific tissues such as floral organs, pericarp and young leaf tissue (Martin et al., 1991; Goff et al., 1992; Quattrocchio et al., 1993; de Majnik et al., 1998). When $ci$, $r$ or $b$ mutations were present, the levels of messenger RNA (mRNA) and enzymes encoded by $DFR$ and $CHS$ were not detectable (Dooner and Nelson, 1979; Dooner, 1983; Ludwig et al., 1989).

Most of these regulatory genes encode transcription factors that act as activators or repressors of DNA transcription or in protein-protein interactions. These regulatory factors belong to the ‘MYC’ and ‘MYB’ protein families that display conserved amino acid motifs such as a basic helix-loop-helix (bHLH) or a basic leucine zipper (bZIP) (Tamagnone et al., 1998; Weisshaar and Jenkins, 1998). The proteins themselves may be further subject to spatial, environmental and developmental regulatory events (Goff et al., 1992; Devic et al., 1999). The MYB loci such as $CI$, $Cl-I$ (Cone et al., 1986; Pas-Ares et al., 1990) and $Sn$ (Tonelli et al., 1991) that regulate biosynthesis in the anthocyanin pathway are distinct from the $P$ locus, reported by these authors for expression systems in both legume and maize. Regulatory proteins encoded by $CI$ (and
its variant, \textit{CI-I}) and by \textit{Sn} are thought to affect later biosynthetic steps, by acting specifically on expression of \textit{DFR} (Sainz et al., 1997). Transient expression experiments in petunia mutants were used to illustrate that ectopic expression of \textit{CI} and \textit{Le} ('leaf colour', from the \textit{R} gene family) was sufficient to induce activity from the promoter of \textit{DFR} in a genotype where the \textit{DFR} gene was ordinarily silent (Quattrocchio et al., 1993). In contrast to these mutants, developing leaves in wild-type petunia normally contain dihydroflavonols and also produce anthocyanins (Quattrocchio et al., 1993). It is evident from these studies that regulation of \textit{DFR} is a key target for controlling the production of flavonoid-based pigments in plant tissue.

\textbf{2.4.3 Seed colour inheritance in \textit{Brassica} species}

The inheritance of seed colour of \textit{Brassica} species may be considerably more complex than would be indicated by the current literature. There are few reports on the regulation of seed coat pigmentation patterns, or on the identity of the compounds contributing to seed coat pigmentation. \textit{Arabidopsis thaliana} is the closest relative to \textit{Brassica} species in which seed coat mutants have been isolated and the regulation of pigmentation has been studied (Kubasek et al., 1992; Shirley et al., 1995). Lack of pigmentation in the leaves and seed coat of \textit{Arabidopsis} has been associated with either defective structural genes or changes to regulatory loci such as \textit{ttg} or \textit{tt8} (Table 2.1). A detailed examination of the regulatory genetic factors in these mutants has the potential to explain the nature of the regulatory protein families in the Brassicaceae.

In research using transgenic plants, the regulatory genes \textit{R/B} and \textit{CI} that ultimately affected anthocyanin biosynthesis in maize have been used to study pigment production in \textit{Arabidopsis} and tobacco (\textit{Nicotiana tabacum} L.) (Lloyd et al., 1992).
Table 2.1  Characteristics of the *Arabidopsis transparent testa* (*tt*) mutants

<table>
<thead>
<tr>
<th>Locus</th>
<th>Enzyme Activity Affected</th>
<th>Flavonol content²</th>
<th>Pigmentation³</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>quercetin</td>
<td>kaempferol</td>
</tr>
<tr>
<td>Structural genes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>tt3</em></td>
<td>Dihydroflavonol 4-reductase (DFR)</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td><em>tt4</em></td>
<td>Chalcone synthase (CHS)</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td><em>tt5</em></td>
<td>Chalcone isomerase (CHI)</td>
<td>−</td>
<td>−/+</td>
</tr>
<tr>
<td><em>tt6</em></td>
<td>Flavonol synthase (FS)</td>
<td>−</td>
<td>−/+</td>
</tr>
<tr>
<td><em>tt7</em></td>
<td>Flavonol 3’-hydroxylase (F3’H)</td>
<td>−</td>
<td>++</td>
</tr>
</tbody>
</table>

Regulatory genes

| *tt8* | Affects *DFR* expression | ++        | ++         | +      | yellow |
| *ttg* | Affects *DFR* expression, seed coat traits and trichomes | ++        | ++         | −      | yellow |

Wild-type  ++        ++         +      brown

¹ from data in Shirley *et al.*, 1995 and Shirley, 1996

² for flavonols present: +, reduced amount relative to wild type levels (++); −, absent; +/−, trace amounts

³ for pigment present other than chlorophyll; identified as an ‘anthocyanidin’ in the seeds and leaves; +, present; −, absent
Pigment biosynthesis was activated by by ectopic expression of these maize regulatory genes in transgenic plants. Anthocyanins were produced in the leaves and pigments were deposited in the seed coat of *Arabidopsis*.

In *Brassica rapa*, a species in which the inheritance of seed color has been widely studied, the source of germplasm may have a bearing on the number of genes found to control the seed colour trait. Schwetka (1982) examined seven yellow-seeded accessions of *B. rapa*, two from a yellow sarson background, (a yellow-seeded, self-compatible subspecies from India), five European accessions from the Institute of Agronomy and Plant Breeding (Göttingen, FDR) and one brown-seeded accession from this Institute. His results demonstrated that up to three genes may control seed colour in *B. rapa*, including a gene that specifically controls hilum colour. Epistatic effects from two of the seed colour genes were observed. This account is one of the few that mentions pigmentation patterns (hilum colour) in the genus *Brassica*.

In another study, however, a single gene was reported to control seed colour, with brown completely dominant over yellow, in progeny of *B. rapa* yellow sarson crossed with reddish-brown ‘toria’ lines (self-incompatible, brown sarson germplasm from India) (Ahmed and Zuberi, 1971; Hawk, 1982). However, three distinct colours of pigmented seed coat (dark red-brown, red-brown, yellow-brown) had been described earlier when Mohammad *et al.* (1942) made crosses with yellow sarson and toria. Yellow seed was produced only when the three loci responsible for the brown seed pigmentation were all in a homozygous recessive state. Since yellow sarson and toria are not pedigreed cultivars, the seed used in these experiments was likely to be genetically distinct from the seed used by Ahmed and Zuberi (1971) or Hawk (1982).
When three cultivars of *B. rapa* developed in Canada (cv Echo, cv Polar and cv Torch) and Swedish material (cv Arlo) were used as the brown-seeded parents in crosses with yellow sarson, dominance at two loci produced dark brown seed (Stringam, 1980). Dominance at one locus (*Br*) resulted in brown seed whereas dominance at the *Br* locus in the presence of recessive *br* produced light-brown seed. Yellow seed was produced when both loci were recessive (*br*/*br*).

In *B. napus*, the number of genes controlling seed colour is estimated to be at least three, again apparently depending on the accessions used in the investigation (Shirzadegan, 1986; Hou-Li *et al.*, 1991; Van Deynze and Pauls, 1994). In crosses between a selection of cv Tower, and Swedish material or other European accessions of *B. napus*, seed colour was reported to be controlled by three genes (*Bl*, *Bl*, *Bl*) (Shirzadegan, 1986). Recessive alleles at all three loci were required to produce yellow seed. When *Bl* was homozygous dominant in these crosses, there were epistatic effects on the other loci, and black seed was produced. Brown seed was expressed under all other genotypic conditions. The contribution of regulatory factors similar to those described for legumes and the possible inheritance patterns to account for these epistatic effects for either *B. napus* or *B. rapa* remain unknown.

In *B. juncea*, yellow seed colour is a recessive trait reported to be controlled by two genes (Vera *et al.*, 1979; Vera and Woods, 1982). Control of seed colour in this species was proposed to be similar to that in *B. napus*, in that both loci must be recessive for the plants to yield purely unpigmented seed. There are no published accounts of seed coat pigmentation patterns or hilum colour in *B. juncea*.

Studies with *B. carinata* reported that one locus controls seed coat pigmentation, but that this was subject to a repressor mechanism that inhibits seed coat
pigmentation (Getinet and Rakow, 1997). This is the only study in the Brassica breeding literature where a regulatory factor has been postulated to account for repression of seed coat pigments to produce a yellow-seeded phenotype with a dominant mode of inheritance. However, the seeds in B. carinata have pigment deposited in spots and around the hilum and the F₁ seed was noted to be a darker yellow colour, so the factors involved in the repression of seed coat pigmentation may be more complex than the single one reported.

2.5 Co-pigments: colourless phenylpropanoids and flavonoids

Phenylpropanoids such as the cinnamic acid esters, absorb most strongly in the short-wave ultra-violet (UV) region (210-350 nm) while flavanones, flavonols and flavones have two to three absorption maxima between 240 nm – 270 nm and 300 nm – 450 nm (Jurd, 1962; Mabry et al., 1970). In UV-light, these compounds are fluorescent; however, under natural daylight, they are invisible to the human eye (Kevan et al., 1996). Nonetheless, in terms of visible pigmentation, there is evidence that colourless flavonoids enhance pigmentation in the visible range or brighten apparently white or light-coloured tissues (Harborne, 1984; Harborne and Williams, 1995; Kevan et al., 1996). Colourless phenolic acids can be co-pigmentation compounds as well. For example, chlorogenic acid changed the colour of anthocyanins from blue-red to yellow-orange or purple-red and increased the intensity of pigments extracted from wine grape (Vitis vinifera L.) (Liao et al., 1992).

2.5.1 Mechanism of co-pigmentation for visible pigments

The mechanism of co-pigmentation has been described as a molecular interaction that occurs between visible pigments (e.g. anthocyanins) and invisible compounds (Mazza and Brouillard, 1990; Liao et al., 1992). Co-pigmentation
compounds increase the colour intensity of anthocyanins and increase the wavelength of maximum absorbance (bathochromic shift). The magnitude of the co-pigmentation effect is influenced by the pH of the solution and the hydration of the pigment. Hydrophobic stacking of the co-pigment and the charged anthocyanidin molecules displaces the equilibrium of the hydration in the anthocyanin, causing the bathochromic shift (Mazza and Brouillard, 1990; Davies and Mazza, 1993).

2.5.2 Occurrence of co-pigment effects on visible pigmentation

Co-pigmentation effects have been discovered in flower and seed coat tissues in diverse genera (e.g. Compositae, Malvaceae, Fabaceae) with hydroxybenzoyl- and malonyl- conjugated anthocyanins and similarly conjugated flavones and flavonols (Harborne and Williams, 1995). Colourless phenolic compounds such as chlorogenic acid, caffeic acid and rutin (a flavonol glycosylated with glucose and rhamnose) exerted a co-pigmentation effect on pelargonidin 3-glucoside and malvidin 3, 5-diglucoside extracted from Monarda fistulosa L. (Asen et al., 1971). Colourless caffeic glycosides were found associated with anthocyanins in the aleurone of purple seeds of sunflower (Helianthus annuus L. Hort.) (Harborne and Williams, 1995).

In the Brassicaceae, seed coat co-pigmentation has been examined in pigment mutants of Arabidopsis (Shirley et al., 1995). The flavonol content (quercetin and kaempferol) of Arabidopsis seeds was reduced when mutations occurred to change the enzyme activity of CHI (tt5 mutant) or CHS (tt4 mutant), the first and second biosynthetic steps in the flavonoid pathway leading to condensed tannins. Plants with different tt mutations either displayed yellow or pale brown seed and a lower amount of anthocyanin in the leaves but the flavonol content was normal, although only kaempferol was detected in certain mutants (Table 2.1, tt5, tt6 and tt7). Under UV-
light ($\lambda_{325}$), the fluorescence of the floral parts was attenuated by normal levels of flavonols in wild-type (WT) Arabidopsis compared to the tt4, tt5, tt6 and tt7 mutants with reduced or no flavonol (Koornneef et al., 1982; Shirley et al., 1995). Myricetin, another flavonol, was not detected in the leaves or seeds of either the mutants or WT plants.

In yellow-seeded bean cultivars, co-pigmentation flavonoids were found unaccompanied by anthocyanins. Seed coats of yellow-seeded dry bean (Phaseolus vulgaris L. cv Prim), were found to contain mostly flavonols and no condensed tannins or anthocyanins (Beninger and Hosfield, 1998). High-performance liquid chromatography (HPLC) was used to isolate and identify these flavonols as glycosides of kaempferol, the only flavonoids found in the cultivar. Concentrations of these compounds were considered quite high (49.9 to 58.5 mg 100 g⁻¹ of fresh whole-bean weight) and were considered responsible for imparting the yellow colour to the seed coat (Beninger and Hosfield, 1998).

2.6 Lignin in seed coats

Lignin is a complex polymer formed by cross-linking different hydroxy-cinnamyl units: $p$-coumaryl, coniferyl and sinapyl alcohols. These monolignols are derived from phenylpropanoids ($p$-coumaric acid, $p$-coumaroyl-CoA) and are named according to the substitution on the phenol ring: $p$-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) moieties, respectively (Sarkanen and Ludwig, 1971). Polymeric lignins are hence classified according to this nomenclature, e.g. guaiacyl lignin, syringyl lignin. The pathway was recently revised and it has been found that syringyl lignin is derived from 5-hydroxy-feruoyl CoA or 5-hydroxy-coniferaldehyde rather
than directly through a sinapic acid precursor (Humphreys et al., 1999) (Figure 2.5).

Lignin is deposited in conjunction with proteins, polysaccharides, suberin and phenolic esters, such as ferulates, during cell wall synthesis (Taiz, 1984; Lewis and Yamamoto, 1990; Boudet et al., 1995; Boudet, 1998). Although it has been suggested that lignins are deposited only in secondary cell walls after the cells cease to enlarge, recent evidence from maize indicates that small amounts of lignin are deposited in developing primary cell walls as well (Müsel et al., 1997). Müsel et al. (1997) proposed that lignification of developing cell walls is indicative of a role in primary plant metabolism.

There is renewed interest in discovering a method to regulate lignin biosynthesis since a reduction in the amount of lignin in crop residues for animal feeds or for the pulp and paper industry would be commercially valuable (Lewis and Yamamoto, 1990; Douglas, 1996; Whetten et al., 1998). Based on experiments modifying lignin formation in transgenic tobacco, Sewalt et al. (1997) suggest that plants with an increased syringyl/guaiacyl ratio might be more digestible and that such plant residues may degrade more readily.

Traditional assays such as acid detergent fibre and neutral detergent fibre only provide a measure of residual plant material, following severe chemical treatment. These determinations may not prove that lignin is actually present in the residue (Lewis and Yamamoto, 1990). Other research indicates that proteins might co-precipitate with the monolignols and interfere with the accuracy of lignin assays (Whitmore, 1978b). Since lignin forms a matrix with cell wall-bound carbohydrates and protein due to the
Figure 2.5  The biosynthesis of lignin.
Derivation of the monolignol units which contribute to the lignin polymer.
Abbreviations (in red) indicate enzymes as follows:

C3H, cinnamoyl-3-hydroxylase; COMT, caffeoyl-O-methylexferase; F5H, feruoyl-5-hydroxylase; OMT, O-methylexferase; 4-CL, 4-coumaroyl CoA ligase; CCoA3H.

*p*-coumaroyl-CoA 3-hydroxylase; CCoAOMT, caffeoyl-CoA O-methylexferase; CCR, cinnamoyl-CoA-reductase; CAD, cinnamoyl alcohol dehydrogenase.

Adapted from Humphreys et al. (1999).
hydroxyproline-rich cell wall (Vanetten et al., 1961), these readily form covalent bonds with isolated lignin monomers (Whitmore, 1978a). Cinnamic acid derivatives (e.g. ferulates and coumarates) are also sometimes found associated with lignin in both secondary and primary cell walls (Taiz, 1984), and have been suggested to be a source of additional problems in accurate lignin estimation.

Although lignin is one of the most indigestible components of canola meal (reviewed in Appendix C), it has been isolated and measured specifically (as 'Klason' lignin) in canola hull material in only one report (Stringam et al., 1974). Otherwise, lignin concentration in canola hulls has been reported from estimates that were actually traditional crude fibre procedures (Mitaru et al., 1982; Naczk et al., 1998). Lignin determination rather than fibre determination would be more useful in relating seed meal to digestibility because the identity of the specific compound associated with indigestibility would be measured. The determination of fibre and its relationship to lignin is discussed further in the methodology review section, Appendix B.2.

2.6.1 The contribution of lignin to seed coat pigmentation

Secondary thickening of the palisade layer in seed coat tissue of several dark-seeded Brassica species results from increased lignification of the cell wall material. Secondary thickening is lacking in yellow-seeded samples (Bouman, 1975; Buth and Ara, 1981). These researchers suggest that the increased lignification contributes to the dark appearance of the dark-seeded Brassica seeds. Lyshede (1982) observed that as the amount of lignified tissue increased, the transparency of the seed coat decreased, making the seed appear darker. He showed that thickened ridges from compressed palisade cells contribute to the dark appearance of seed coats in Japanese radish (Raphanus raphanistrum L.) compared to the cell walls in the surface and palisade
layers of seed coats in thinner-walled European radish (*R. sativus* L.) which were more transparent.

There is other evidence linking seed coat pigmentation and lignin. Since lignin is a major component of fibre (Spiller, 1986), there is a correlation between seed coat pigmentation in *Brassica* *spp.* and the concentration of fibre in *Brassica* seed meal (Simbaya *et al.*, 1995). Significant differences were found in fibre concentration between yellow- and black-seeded *B. rapa* lines (Stringam *et al.*, 1974). Separate analyses of seed coat and embryo tissue showed that at least 90% of the crude fibre was associated with seed coat material and that the fibre concentration was lower in seed coats of yellow-seeded material. In contrast, fibre concentration in the embryo tissue was lower in brown-seeded *B. rapa* compared to the embryo tissue from the yellow-seeded lines on a per seed basis.

By using histological methods, it was determined that there are significant differences in seed coat thickness in *B. rapa* (Stringam *et al.*, 1974). Thickness in yellow-seeded lines averaged 259 µm, compared with 318 µm in black-seeded lines. The cells in the palisade layer of the seed coats from yellow-seeded *B. rapa* were one-half to two-thirds the size of those from brown-seeded material, thereby accounting for the pronounced differences in thickness (Vaughan and Whitehouse, 1971; Stringam *et al.*, 1974). This confirmed earlier observations of several Brassicaceae that yellow-seeded cultivars had thinner seed coats than dark-seeded cultivars although seed coat thickness had not been specifically measured (Vaughan, 1960).

Differences in fibre and seed coat thickness have been associated with seed colour in other crop species. Fibre is lower in yellow-seeded flax lines relative to brown-seeded cultivars (Saeidi, 1997). Also, as in yellow-seeded *Brassica* *spp.*,
yellow-seeded flax has thinner seed coats than brown-seeded cultivars (Flax Council of Canada, 1996). Unpigmented legume seeds display a similar association of these traits. In lima bean (*Phaseolus lunatus* L.), the white cultivars have thinner seed coats than pigmented cultivars (Kannenberg and Allard, 1964).

2.7 Molecular techniques to analyze the structure and function of genes

2.7.1 RFLP-based analysis of plants for gene function

Analysis using RFLP patterns is based on the detection of DNA sequence variation within fragments that are derived from genomic DNA by restriction enzyme digestion and separated by agarose gel electrophoresis (Southern, 1975). RFLPs can arise as a result of mutations associated with the various alleles of a gene (Soller and Beckman, 1983). Seed coat pigment variation and patterning in soybean has been investigated by RFLP analysis. The *l* locus, that controls pigmentation in soybean seed coats, was identified by classical Mendelian genetics (Bernard and Weiss, 1973), but at that time, the structural gene was unknown. Subsequently, RFLP analysis of near-isogenic lines, has shown that variation at this locus corresponds to mutations in the *CHS* promoter and coding regions (Wang *et al*., 1994). Duplications and deletions of specific *CHS* sequences in this multigene family control the flux of phenylpropanoids into the flavonoid pathway (Akada and Dube, 1995; Todd and Vodkin, 1996).

Similarly, using a doubled-haploid population of *B. napus*, DNA fragments linked with seed colour were sequenced and identified as fragments of the *CHS* gene (Van Deynze *et al*., 1995).

2.7.2 Transcript analysis to determine gene expression

Variability of a trait can often be explained by monitoring the production of mRNA by Northern blots or by the more sensitive method, reverse-transcriptase PCR
(RT-PCR). In *A. thaliana*, gene transcripts from the *tt* mutants were monitored by Northern blots (Shirley *et al.*, 1995; Kubasek *et al.*, 1998). The lack of pigment in the seed coat in several of these mutants was attributed to defective genes which failed to produce a functional gene transcript (table 2.1, section 2.4.2) However, in one CHS mutant, *tt4*, the level of CHS mRNA was similar to that in the wild-type but no flavonoids were produced (Shirley *et al.*, 1995). When enzyme activities were measured, the *tt4* mutant was found to be missing CHS activity, although the other flavonoid enzymes in this mutant were functional.

Flavonoid gene expression in carnation (*Dianthus caryophyllus* L.) has also been monitored by Northern hybridization (Mato *et al.*, 2000). In white-flowered cultivars, *F3H* or *DFR* transcripts were reduced or missing. RT-PCR confirmed that *DFR* as well as *AS* mRNAs were severely reduced in white-flowered cultivars and that *F3H* was absent. In other gene expression research, transcripts from genes that control flowering in *B. rapa* were difficult to monitor by Northern hybridization. However, these were detected by RT-PCR (Blahut-Beatty, 1999). In both of the above studies, actin (a constitutively produced mRNA), was used to establish that quantitative aliquots of RNA were used for both Northern hybridization and to produce the cDNAs for RT-PCR. Monitoring constitutively expressed transcripts, such as actin, has been used in a number of gene expression studies employing RT-PCR (Yu *et al.*, 1998; Ride *et al.*, 1999; Shimizu *et al.*, 1999). Other transcripts that have also been used for RNA quantitation include α-tubulin (Sun *et al.*, 1999), β-tubulin (Aranda *et al.*, 1999), 18s rRNA (Marsh and Kaufman, 1999) ubiquitin (Zhuo *et al.*, 1999), heat shock protein, *CaHSP1* (Rigola *et al.*, 1998) and protein kinase, *atpk1* (Brzeski *et al.*, 1999).
CHAPTER 3

MATERIALS AND METHODS

3.1 Source of the B. carinata germplasm and seed of selected Brassicaceae

Brassica carinata accessions were contributed by Dr. Gerhard Rakow, Agriculture and Agri-Food Canada, Saskatoon, Canada (AAFC). These accessions were from Canadian-grown, bulked material that originated from Ethiopian lines, provided to AAFC by Dr. A. Getinet, Plant Genetic Resource Centre, Ethiopia (PGRC/E). Samples of the accession PGRC/E 21164 were used in previous research at AAFC to determine the genetic control of seed coat pigmentation and associated meal quality traits as well as for introgressing the unpigmented seed coat character into B. napus (Getinet, 1986; Rashid et al., 1994; Simbaya et al., 1995).

Pure breeding yellow- and brown-seeded lines of B. carinata PGRC/E 21164 were developed from one yellow- and one brown-seeded S1 plant propagated in growth-chamber and greenhouse facilities (details, Appendix D.1). A yellow-seeded S3 line (the ‘Y’ line) and a brown-seeded S2 line (the ‘B’ line) were used for detailed chemical and molecular analyses (Figure 3.1). The production of field-grown material is described in Appendix D.5. The source of samples of other Brassicaceae species for histochemical, condensed tannin and lignin determinations is described in Appendix D.6.
Figure 3.1 Development of *Brassica carinata* PGRC/E 21164 Y line and B line near-isogenic germplasm.
3.2 Reagents

Water and organic solvents (EM Sciences) were of HPLC-grade; authentic standards were obtained from Apin Co. (London, UK.) and dissolved in methanol (MeOH), unless otherwise indicated; other chemicals were reagent grade or better. In the extraction and analyses of DNA and RNA, chemicals were molecular biology-grade from Sigma unless otherwise mentioned; disposable plastics were polypropylene (Fisher); the molecular marker ladder for agarose gels was ‘1 Kb-Plus’ (Gibco-BRL) unless otherwise specified. Nucleic acids were transferred to Hybond N+ membranes (0.45 μm, Amersham Pharmacia) for hybridization in a Hybiad oven (InterScience, Markham, ON). Pre-prepared commercial reagents and kits are identified where used. Sterile, HPLC-grade water was used for all aqueous solutions. Diethylpyrocarbonate (DEPC) was used to inactivate RNAses in the water used for reagents; these reagents were stored in sterile, oven-baked (240 °C) glassware. Filter-pipette tips (Mandel) were used for all RNA work. Radioisotope (α-32P) was obtained from Amersham (specific activity, 3000 Ci/mmol dCTP). Scintiverse fluor (Fisher) was used in liquid scintillation spectroscopy.

3.3 Equipment

Glass or teflon-coated (PTFE) lab-ware was used for analytical organic extractions of seed preparations. For centrifugation, a swinging-bucket rotor (RTH-750) in a Sorvall (Model RT-7) was used at 2537 g unless otherwise noted. Three methods (specified where applicable) were employed for powdering plant tissue, (a) grinding in Oakridge centrifuge tubes (Nalgene) using interchangeable probes (0.2 cm and 1.0 cm diameter) attached to a Polytron (Brinkman); (b) milling (Spex Certi-Prep, Model 8000) with 5.0 mm diameter stainless steel beads in capped vials; or (c) grinding
by mortar and pestle with liquid nitrogen. Rotary-evaporation took place at 40°C under vacuum using a Büchi model R-114. Thin layer chromatography was conducted using 'Avicell' microcrystalline cellulose TLC plates (plastic-backed, Macherey-Nagel). Samples were filtered through a Buchner funnel lined with Whatman #4 paper, unless otherwise specified.

Amplification by polymerase chain reaction (PCR) was accomplished using a programmable thermal-cycler, (model PTC-200, MJ Research Inc., Watertown, MA). A Bio-Rad electrophoresis system was used with agarose gels (2%) to separate nucleic acids. Ethidium bromide-stained (EtBr) gels were illuminated by UV light (λ₃66) and photographed with a Stratagene gel documentation system (Eagle Eye II; 'Eaglesight' software [ver. 3.2]). DNA was quantified by fluorometry (Fluoroskan II, Stratagene) with a fluorescent dye (0.2 μg μL⁻¹, Hoechst 33258, Sigma). In hybridization experiments, background radioactivity was monitored by a hand-held radiation detector (Geiger-Mueller PUG 1AB, Technical Associates Inc., Canoga Park, CA). The specific activity of radioactive hybridization probes was measured using a Canberra-Packard 2200 liquid scintillation counter.

3.3.1 Analytical RP-HPLC and mass spectrometry

RP-HPLC 'A': Hewlett-Packard (HP) Series 1100 (San Diego, CA) equipped with a model 1050 autosampler, a programmable photodiode array (PDA) detector, a heated (30°C) reversed-phase (RP) LiChrospher C₁₈ column (E. Merck, 4.0 x 125 mm, 5 μm particle size) and a water-acetonitrile solvent system modified with 0.2% acetic acid (HOAc) (v/v). HP ChemStations software (ver. 6.02) was used to control the instrument and analyze data.
**RP-HPLC 'B':** Waters 2690 'Alliance' (Milford, MA) equipped with an autosampler, a programmable 996 PDA detector, a heated (30 °C) Symmetry RP C$_{18}$ column (Waters, 3.0 x 150 mm; 5 μm particle size) and a water-acetonitrile solvent system modified with 0.05% trifluoroacetic acid (TFA) (v/v). Instrument separations and data analyses were controlled using Waters Millenium 32 software (ver. 3.2).

**RP-HPLC 'C':** Liquid chromatography-tandem mass spectrometry (LC-MS-MS) was performed using a ‘Quattro LCZ’ (MicroMass Co., Manchester, UK) bench-top system with ‘Z-spray’ interface and an Alliance RP-HPLC system configured as described for RP-HPLC ‘B’, substituting a water-acetonitrile solvent system modified with 0.1% formic acid. Sample ionization was achieved using the atmospheric pressure chemically-induced ionization technique (APCI) with instrument settings for parent molecular ion and daughter ion analyses as summarized (Table 3.1); the instrument was controlled using MassLynx software (ver. 3.4).

### 3.4 Histochemical detection of anthocyanins and condensed tannins

Seed coat tissue was removed by hand from seed samples soaked for 1-2 h in distilled water to soften the seed coat, with the exception of *A. thaliana* seed, which was used whole. Histochemical stains (BuOH-HCl [70:30, v/v], 1% HCl in MeOH, EtOH-HCl [2:1, v/v] and freshly prepared 1% vanillin in 6N HCl) were used according to the methods of Skadhaug et al. (1997) and Lees et al. (1993). The initial appearance of unstained seed coat specimens was observed first as wet mounts, using a dissecting microscope (Nikon MKII, 1.5x to 6x). Seed coats were again examined immediately upon staining to determine the presence of anthocyanins, and subsequently every hour for the next 6 h to monitor the development of red-stained
Table 3.1  Mass spectrometry settings for analyzing phenylpropanoids and flavonoids by APci\(^1\) in a Z-spray interface

<table>
<thead>
<tr>
<th>Instrument Component</th>
<th>Parameter (Unit)</th>
<th>Setting</th>
<th>APci(^{\text{Parent ions}})</th>
<th>APci(^{\text{Daughter ions}})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corona discharge pin</td>
<td>voltage (V)</td>
<td>3.84</td>
<td>3.84</td>
<td></td>
</tr>
<tr>
<td>Cone</td>
<td>voltage (V)</td>
<td>25.0</td>
<td>25.0</td>
<td></td>
</tr>
<tr>
<td>Source Block</td>
<td>temperature (°C)</td>
<td>150</td>
<td>150</td>
<td></td>
</tr>
<tr>
<td>APci probe</td>
<td>temperature (°C)</td>
<td>500</td>
<td>500</td>
<td></td>
</tr>
<tr>
<td>Nebuliser (nitrogen(^2))</td>
<td>gas flow (l min(^{-1}))</td>
<td>7.02</td>
<td>7.25</td>
<td></td>
</tr>
<tr>
<td>Desolvation (nitrogen(^2))</td>
<td>gas flow (l min(^{-1}))</td>
<td>2.56</td>
<td>2.50</td>
<td></td>
</tr>
<tr>
<td>Analyser</td>
<td>vacuum (mBar)</td>
<td>7.4 \times 10(^{-6})</td>
<td>1.6 \times 10(^{-5})</td>
<td></td>
</tr>
<tr>
<td>Collision cell (argon(^3))</td>
<td>vacuum (mBar)</td>
<td>3.6 \times 10(^{5})</td>
<td>3.7 \times 10(^{-4})</td>
<td></td>
</tr>
<tr>
<td>Data acquisition</td>
<td>Scan range (m/z)</td>
<td>100-500</td>
<td>100-350</td>
<td></td>
</tr>
</tbody>
</table>

\(^1\) in negative and positive ion modes
\(^2\) Ultra-high purity
\(*\) for all phenylpropanoids and flavonoids, except flavonols (settings reported in results)
tissue. Examinations were conducted at 72 and 96 h to detect final changes in colour. For photography at higher magnifications (25x and 62.5x), fresh and stained seed coat tissue was rinsed and mounted in water under a cover slip on microscope slides. Photographs were taken under brightfield conditions with an automated camera mounted on a Nikon Optiphot compound microscope. Each observation was repeated four times, once with each reagent. Seeds from *A. thaliana* were placed on a microscope slide indented with a well and all histochemical operations were performed while observing the seeds under magnifications of 2.5x and 25x (dissecting and compound microscopes).

Embryonic tissue was examined in only one species: *Eruca sativa* SRS 2681. Immediately after the seed coat was removed, the embryo and seed coat tissues were flooded with vanillin-HCl on a microscope slide and observed with a dissecting microscope. The experiment was repeated with BuOH-HCl and the seed coat tissues remained in the histochemical stain for 5 d.

### 3.5 Quantitative PVPP-BuOH-HCl assay for extractable condensed tannin

Concentration of extractable condensed tannin was quantified using the PVPP-BuOH-HCl assay of Watterson and Butler (1983), modified by Skadhaug et al. (1997) (assay details, Appendix B.1.3). Replicate samples of whole seed (six, 200-mg each) from selected Brassicaceae and from each generation of the Y and B lines of *B. carinata* PGRC/E 21164 were analysed. Five concentrations of purified condensed tannin polymer (sainfoin, [Koupai-Abyazani et al., 1992]), included in every set of assays, were used to construct a standard curve. Sainfoin polymer was used as a standard because there was no purified polymer available from any species of *Brassica*. 43
Absorbance readings (AU) of the extract supernatants were taken by scanning from 190 nm to 800 nm (Beckman DU-5). The control (no sainfoin polymer present) was used as the reference blank, followed by the standards and samples. The data were analyzed by the baseline subtraction method described by Skadhauge et al. (1997), where absorbance due to condensed tannin is calculated by the height of the absorption peak at 550 nm minus the baseline scan from the zero control. The data for each specimen were averaged (n= 6) and the standard error of the means (SE) calculated.

3.6 Anthocyanin content in seeds and seedling leaf tissue

The presence of anthocyanins in seed and seedling leaf tissue was determined by the iso-amyl alcohol method of Harborne (1984). Seedlings were grown under the conditions described in Appendix D.4. Anthocyanin extraction was performed on leaf tissue and seeds of the Y and B lines of B. carinata PGRC/E 21164. Leaf tissue (2 g) was used directly in the extraction; seeds (2 g) were initially ground to fine particles in liquid nitrogen with a pestle and mortar for extraction. The iso-amyl alcohol fractions were rotary-evaporated under high vacuum (≤5 mBar) at 40°C, re-dissolved in 1% HCl-MeOH (v/v) and the absorbance spectrum between 190 and 700 nm measured (Beckman, DU-5). Methanol-HCl (1%) was used as a reference blank and cyanidin (Sigma) and pelargonidin (Sigma) were used as authentic standards. One- and two-dimensional thin layer chromatographic (1D-, 2D-TLC) separations were conducted on cellulose TLC plates developed in 15% HOAc (first dimension or for 1D-TLC) and Forestal (conc. HCl: HOAc: water [3:10:30]) (second dimension) or in freshly prepared n-butanol: HOAc: water (4:1:5, v/v, upper phase, BAW) (Harborne, 1984; Markham, 1982; Mabry et al., 1970). Authentic standards (cyanidin [Sigma], delphinidin
[Aldrich], malvidin [Aldrich], pelargonidin [Sigma], pãœonidin [Apinl]) were included on 1D-TLC plates. Resolved coloured spots from 2D-TLC were scraped off the plate and eluted with 1% HCl in MeOH. The absorbance (190 to 700 nm) of each eluant was measured as described previously.

3.7 Determination of thioglycolic lignin

Thioglycolic lignin concentration was estimated according to Whitmore (1978a), as modified by Campbell and Ellis (1992) for small sample volumes. This thioacetolysis method was tested for the effects of compounds unrelated to lignin that exhibit maximal absorbance in the region of 280 nm (e.g. sinapine), or that may bind lignin (e.g. hydroxyproline-rich proteins, cell wall debris) (Whitmore, 1978b). One B. carinata seed sample ([A] S-67, 200 mg) was compared with six control samples ([B] gelatin (Knox), a source of hydroxyproline-rich protein (5 mg), [C] bovine serum albumin (5 mg), [D] freeze-dried B. carinata mature stem tissue (200 mg) [E] sinapine standard (gift from Dr. Ian McGregor, AAFC, Saskatoon, Canada) (2 mg) [F] a mixture of lignin standard (5 mg) plus gelatin (7.5 mg), [G] lignin standard (milled spruce lignin, gift of Dr. P. Watson, Pulp and Paper Institute of Canada (PAPRican), Vancouver, Canada), [5 mg]).

Thioglycolic lignin determination was conducted with 200-mg ground (Polytron) samples of whole seeds of the Brassicaceae and seeds from each generation of the B. carinata PGRC/E 21164 material from which the Y and B lines were developed. The final thioglycolic lignin pellets, resuspended in 1 M NaOH, were diluted 50x with 1M NaOH and the absorbance (λ280) was measured on a spectrophotometer (Beckman DU-5) (1 M NaOH, reference blank). A standard curve
was constructed from absorbance measurements of five concentrations of lignin standard, included in each sample set. The results for lignin concentration were averaged and SE were calculated (n=6).

Thioglycolic lignin concentration was determined in dissected seed coat and embryo material from B. carinata PGRC/E 21164 Y line generations (S₁, S₂, and S₃) and B. carinata PGRC/E 21164 B line generations (S₁ and S₂). The objective was to determine the proportion of lignin occurring in the seed coat with respect to the whole seed. A 200 mg sample of seed was weighed and the seed coat and embryo hand-dissected. Air-dried seed coat and embryo tissues were individually weighed after dissection. Lignin determinations were performed as for whole seed material.

3.8 Extraction and fractionation of colourless phenolics from B. carinata

Mechanically-collected seed coat (Appendix E), hand-dissected seed coat from the Y and B lines of B. carinata PGRC/E 21164, and hand-dissected seed coat from brown-seeded B. carinata cv S-67 were extracted for colourless phenolics. Only preliminary extraction experiments used whole-seed samples. Extraction protocols were based on procedures for phenylpropanoids and flavonoids according to Harborne (1984), Markham (1982), Harborne et al. (1975) and Mabry et al. (1970).

The optimum solvent for isolation of phenylpropanoid and flavonoid aglycones was determined using four concentrations (50%, 60%, 80% and 100%) of hot MeOH (60°C, 10 mL solvent gram⁻¹). Four 5-g whole seed samples from a yellow-seeded (S₁ generation) and a brown-seeded (cv S-67) sample were ground in liquid nitrogen and extracted for 24 h in the MeOH which was heated (60°C) twice for 20 min, at the beginning and the end of the extraction. These methanolic extracts were acidified to a final concentration of 2 M HCl and re-heated to 90 to 95°C for 30 to 40 min to cleave
the O-glycosidic bonds between sugars and the parent flavonoid molecules (aglycones) (Onyilagha, 1994; Harborne, 1984), except for the acidified 100% MeOH preparation, which was heated for 10 min. The remaining MeOH in the extracts was removed by rotary evaporation at 25°C and each preparation was partitioned against ethyl acetate (EtOAc) (2:1, v/v) four times to isolate the aglycones and the four washes were combined. Each preparation of combined EtOAc extracts was concentrated and dried with anhydrous Na₂SO₄. The resulting samples were filtered, reduced to dryness and re-dissolved in 1-mL aliquots of 100% MeOH for evaluation by 1D-TLC.

Hand-dissected seed coat (1.4 g each, freeze-dried tissue, Y and B lines and cv S-67, B. carinata) were extracted as described above, except that the initial solvent was 80% MeOH. The pooled EtOAc extractions of each sample were dried with anhydrous Na₂SO₄, filtered and evaporated to dryness. Each of the EtOAc-soluble residues was re-dissolved in 250 μL 100% MeOH, filtered (0.45 μm [Gelman]) and stored at −20°C until analyzed. This extraction was performed twice on two separate 1.4-g samples of seed coat tissue.

Mechanically-collected seed coat samples (78 g, Y line and 76 g, B line, B. carinata) were subjected to the whole seed extraction protocol using 80% MeOH as the initial solvent. The final, dried EtOAc-soluble extract from the Y line was fractionated sequentially by washing the residue with (1) water, (2) 30% MeOH and (3) 100% MeOH. The residual EtOAc-soluble extract remaining after each wash was taken to dryness by rotary evaporation between each washstep. The B line was analyzed as a total extract only.

Aliquots (2.0 mL) of the unfractionated EtOAc extract from mechanically-collected seed coat of the Y line (before fractionation) and B line were subjected to
basic hydrolysis by heating the 2 mL of extract for 5 min (90°C) with 500 μL of 1 M NaOH followed by a 4-h incubation at 22°C. The extract was adjusted to pH 5.5 – 6.0 with ca. 500 μL of 1 M HOAc, filtered (0.45 μm) and injected into HPLC system ‘B’. The total EtOAc extract from cv S-67 was treated by the same procedure but using smaller volumes of extract and reagents (200 μL extract, 50 μL of 1 M NaOH, 50-60 μL of 1 M HOAc).

3.9 Cellulose TLC analysis

3.9.1 Evaluation of extractions with four concentrations of MeOH

Brassica carinata cv S-67 and yellow-seeded S1 whole-seed extracts (20 μL, each) from the four different MeOH concentrations, were separated on a cellulose TLC plate developed with freshly prepared BAW (Harborne, 1984; Mabry et al., 1970). The capability of each solvent to solubilize phenolics was assessed on the basis of the relative size of separated spots having similar mobilities (the same Rf values), (or number of separated components) when visualized under UV-illumination. The authentic standards, sinapine and sinapic acid (Sigma), were used on the same plate as chromatographic controls since Brassica seed contains large amounts of these two phenolics.

3.9.2 Two dimensional TLC analysis of seed coat extracts

Separations in two dimensions were conducted with extract (30 μL aliquots) from hand-dissected seed coat tissue of B. carinata cv S-67, and the Y and B lines. Cellulose TLC plates were developed in the first dimension with 30% HOAc and in the second dimension with Forestal. An authentic standard (t-cinnamic acid [Sigma], dihydrokaempferol or dihydroquercetin) was also separated on the same plate, in one lane for each dimension. Spots were detected under both short- and long-wave UV-
illumination and fuming with NH₃. The 2D-TLC experiment was repeated twice for each seed coat sample from both extractions of seed coat tissue.

3.10 Analytical HPLC

3.10.1 Analysis of unhydrolyzed MeOH extracts of selected Brassicaceae

Yellow- and brown-seeded samples (200 mg whole seed) of species in the Triangle of U (Appendix A) (B. carinata, B. napus, B. rapa and B. juncea and of brown-seeded samples of B. oleracea and B. nigra) were ground using a Polytron in 1 mL of MeOH, extracted with a final volume of 2 mL 80% MeOH in a waterbath (60°C) for 2 h and centrifuged for 5 min (Eppendorf, fixed angle rotor, 10,000 g). The unhydrolyzed supernatants were immediately filtered (0.45 μm, Gelman) and analyzed by RP-HPLC (system ‘A’, section 3.3.1). Samples were eluted with a water-acetonitrile gradient solvent system modified with 0.2% HOAc (5% to 30% acetonitrile, over 30 min; to 60% acetonitrile, over 5 min; hold 15 min; to 5% acetonitrile, over 5 min) at 1 mL min⁻¹. Authentic standards were separated under the same conditions to create a UV-spectral library for identification of unknown components in the seed extracts.

3.10.2 Analysis of EtOAc-soluble extracts from seed coat preparations

Seed coat extracts from hand-dissected samples of B. carinata cv S-67. Y and B lines were compared for phenylpropanoid and flavonoid content. Extracts (5 and 10 μL aliquots) were analyzed by RP-HPLC (system ‘B’, section 3.3.1) eluted with a 33-min aqueous-acetonitrile gradient (10% acetonitrile, 5 min; 40% acetonitrile, 10 min; hold 40% for 10 min; return to 10% acetonitrile, 5 min; hold 10% acetonitrile, 3 min) at 0.4 mL min⁻¹. A UV-spectral library of authentic standards was constructed by analyzing authentic standards under the same separation conditions. For UV-spectral
identifications, ‘max plot’ chromatograms were generated to show the maximum absorbance of all the components in the extract over a preset wavelength range (200 to 500 nm), rather than UV-absorbance at a single wavelength. The retention time of \( t \)-cinnamic acid in the seed coat extract matrix was established by an addition of the inclusion of \( t \)-cinnamic acid in subsamples of \( B. \ carinata \) Y and B seed coat extracts. These spiked samples were re-analyzed by HPLC under the same conditions as unspiked samples. This experiment was repeated using extracts of mechanically-collected seed coats and dihydroflavanols as the standards.

The three fractions of the EtOAc-soluble extract from mechanically-collected Y line and the total B line seed coat extract were separated according to the same 33-min protocol used for the hand-dissected seed coat. To achieve better resolution for later analyses by LC-MS-MS, a 55-min elution gradient (10% acetonitrile, 10 min: 20% acetonitrile, 15 min; hold 20% for 5 min; 30% acetonitrile, 15 min; return to 10% acetonitrile, 5 min; hold 10% acetonitrile, 5 min) was used. Base-hydrolysed extracts were analyzed using the 33-min gradient with RP-HPLC system ‘B’. Authentic standards were separated under the same conditions using this 55-min gradient and added to the UV-spectral library. Every RP-HPLC analysis of seed coat extract included standards (\( t \)-cinnamic acid and the flavonols, myricetin, quercetin, kaempferol, prepared as a mixture) in the sample set in order to monitor the performance of the equipment.

3.11 Mass spectrometry

Two fractions from the Y line (30% and 100% MeOH) and the unfractionated B line extracts of mechanically-collected samples were analyzed by RP-HPLC (system ‘C’, section 3.3.1). The samples were eluted into the LC-MS-MS (section 3.3.1) using
either the 33-min or 55-min gradient at 0.2 mL min\(^{-1}\) and chemically-induced ionization took place under atmospheric pressure (LC-MS instrument settings, Table 3.1, section 3.3.1). During LC-MS-MS separations of seed coat extracts, there were highly non-polar compounds that did not elute from the HPLC when the gradient reached the maximum concentration of 30% acetonitrile otherwise required for an optimal separation. A 15-min column wash of 95% acetonitrile followed each injection of seed coat extract. In a second experiment to elute such non-polar compounds, the 55-min gradient was programmed for a second sample injection to rise to 50% acetonitrile instead of 30%. Authentic standards were separated under the same conditions with the appropriate 55-min gradient (30% or 50% acetonitrile) in order to determine retention times.

Samples were desolvated with nitrogen gas and analyzed in both negative- and positive-ion mode (NI and PI respectively, with reference to the molecular ion [M-H]\(^-\) or [M+H]\(^+\)). Both total ion current (TIC) chromatograms and selected ion recording (SIR) took place to determine the presence of specific compounds. Fragmentation patterns (daughter ions) were observed by allowing the preselected parent molecular ions to collide with argon in the second quadrupole (collision cell). The resulting daughter ions were detected in the third quadrupole mass analyzer. Fragmentation was optimized by varying the argon pressure in the collision cell.

### 3.12 Analysis of genomic DNA for molecular polymorphisms related to the seed coat pigmentation trait

*Brassica carinata* plants for Southern analysis were grown and freeze-dried as described in Appendix D.2. Genomic DNA was extracted from 38 samples (13 yellow- and 23 brown-seeded S\(_1\) lines and the Y and B lines) using Kirby extraction buffer
(Appendix F.1) according to Covey and Hull (1981). The isolated DNA was resuspended in water and quantified by fluorometry (section 3.3).

Restriction enzyme digestion, agarose gel electrophoresis and blotting to a nylon membrane for hybridization was performed according to Sambrook et al. (1989). Purified DNA samples were diluted with sterile water to provide 15 μg DNA for each restriction digest. Restriction enzymes EcoRI, BamHI and Hind III (High Concentration) (Pharmacia Biotech) (2.5 U of enzyme μg⁻¹ DNA) were incubated with DNA extracts in commercial buffers (Pharmacia Biotech) and digested overnight according to the manufacturer’s instructions. The digested DNA (with the addition of Bromophenol Blue sample loading buffer) and the base pair (bp) marker ladder (λDNA/Hind III [Boehringer-Mannheim]) were separated for ca. 20 h at 40 V on 0.8% agarose gel in 1% TAE buffer, until the dye-front had migrated 20 cm. Gels were stained (EtBr) and photographed prior to depurination in 0.25 M HCl for 30 min and neutralization in 0.4 M NaOH. The DNA in the gel was transferred to a nylon membrane by overnight blotting with 0.4 M NaOH.

3.12.1 Hybridization and autoradiography using radioactive probes

Hybridization of radioactive probes to the immobilized DNA was conducted at 65°C using a blocking solution (4x SETS buffer, 0.1% sodium dodecyl sulfate [SDS], 10x Denhardt's solution, 1% dextran sulfate and 0.5 mg salmon testes DNA, according to Sambrook et al., 1989). The radioactive probe was prepared using ³²P-dCTP and the 'Random Primers DNA Labeling System' (Gibco-BRL) according to the manufacturer's directions. Unincorporated ³²P-dCTP was removed from the reaction by centrifugation (700 g) through a Sephracyl-200HR micro-spin column (Pharmacia).
The heat-denatured probe was hybridized overnight. Probes (PAL, CHS and CAD) were consecutively prepared (Appendix F.2), hybridized to and removed from the same membrane.

Unhybridized probe was removed from the membrane with two 15-min washes (2x SSC, 1% SDS, pre-heated to 65°C) and two 30-min washes (0.2x SSC, 0.01% SDS, 22°C in a cooled hybridization oven) (Sambrook et al., 1989). After the final wash, the membrane was placed in a glass tray and agitated with several changes of fresh 0.2x SSC until radioactivity on the DNA-free part of the membrane was similar to normal background readings (≤200 cpm). Washed filters were exposed to X-ray film in film cassettes with intensifying screens (X-Omat, Kodak) at −80°C for variable lengths of time. The film was developed in an automated developer (Kodak M35A X-Omat Processor).

3.13 Analysis of gene expression

3.13.1 Preparation of total RNA extracts

Seedling leaf tissue, developing seeds and unopened buds for RNA extraction were grown and harvested as described in Appendix D.3 and D.4. Total RNA from samples of seedling tissue (cotyledons, first to fourth leaf stage), floral apices (62 days after pollination [dap]) and developing seed from 5 and 10 dap was extracted using the Plant RNeasy kit (Qiagen), following the manufacturer’s directions. Tissue from 20 and 30 dap was extracted according to the hot phenol method of Verwoerd et al. (1989) with modifications to counteract the viscosity, due to seed storage proteins or polysaccharides of the final preparation (Wilen et al., 1990). Viscous preparations were re-centrifuged for 5 min (10,000 g, Eppendorf) and the supernatant quickly
transferred to a fresh tube. Samples from 30 dap tissue were re-washed with EtOH (final concentration, 70%) followed by precipitation in 95% EtOH, if the viscosity was not satisfactorily reduced.

3.13.2 RNA gel electrophoresis and Northern blotting

Samples of developing seed (0, 5, 10, 20 and 30 dap) total RNA (ca. 20 μg), seedling leaf tissue (first leaf stage; positive control) and RNA markers (0.24 to 9.49 Kb, Gibco-BRL) were separated on denaturing formaldehyde gels (1.2% agarose [Boehringer-Mannheim], 0.66 M deionized formaldehyde, 1x MOPS buffer [3-N-morpholino propanesulphonic acid]) for 3 h at 50 V according to Sambrook *et al.* (1989). For Northern hybridization, the RNA was transferred overnight with 10x SSC to a nylon membrane by capillary blotting. The blotted RNA was fixed to the membrane by UV-induced cross-linking (Stratalinker 1800, Stratagene). The position of the ribosomal RNA (detected under UV-illumination) was marked in pencil on the membrane.

Initially, Northern hybridization was conducted in a hybridization solution (formamide, dextran sulfate, Denhardt's, 1% SDS and salmon testes DNA in SSPE) according to Sambrook *et al.* (1989) preheated to 40 °C. In subsequent experiments, hybridization was performed at 35°C or 30°C. Four probes (actin, CHS, PAL, CAD) were consecutively prepared, hybridized and removed (Appendix F). In a second set of Northern hybridization experiments, Ultrahybe™ (Ambion) was used in consecutive hybridizations with the DFR and actin probes, using the manufacturer's protocol. The probe for DFR hybridizations contained a 400 bp insert that was homologous to a published DFR sequence in *A. thaliana* (Appendices F.2, G.4 and Figure F.1, GenBank
Following hybridization, the membranes were rinsed in consecutive 5 min washes, initially at 35°C (40°C for Ultrahybe experiments) later reduced to 30°C, using 10x SSPE with 1% SDS, followed by 5x SSPE with 1% SDS, 2x SSPE with 0.1% SDS. For blots with Ultrahybe, 2x SSC with 1% SDS followed by 0.2x SSC and 0.1% SDS were used at 40°C. These washes were monitored until radioactivity on the RNA-free part of the membrane was similar to normal background readings (≤200 cpm).

Washed filters were exposed to X-ray film in cassettes with intensifying screens at –80°C for variable lengths of time and the film was developed in an automated Kodak developer. The probe was removed from the membranes by agitation in glass trays with 300 mL hot (70 to 80°C) stripping solution (0.1x SSC and 0.01% SDS).

Densitometry measurements were taken by scanning the autoradiographs of Northern blots having a detectable signal (Bio-Rad Calibrated Imaging Densitometer model 710-GS controlled by ‘Quantity One’ software, ver. 4.1). In each lane the density of the test probe (DFR) was normalized to its corresponding control probe (actin) according to recognized quantification protocols for Northern blots (Conner and Liu, 2000; Suzuki et al., 2000). Northern blots were repeated three times, once for each new set of RNA extracts.

3.13.3 Reverse transcriptase PCR (RT-PCR)

RT-PCR amplification was used to detect differences in the pattern of DFR transcripts in the RNA samples in developing B. carinata seed. In initial experiments, DFR primers (5’ and 3’), based on published A. thaliana sequences (National Centre for Biotechnology Information [NCBI]) within the third exon were used (Appendix G.3). These primers were expected to produce a 400 bp fragment from B. carinata
mRNA or genomic DNA. In subsequent experiments, the 3' primer (21 bp) was
designed within the first exon of DFR from A. thaliana (GenBank, AB007647) and the
5' primer (23 bp) was based on nucleotide sequence data from B. carinata, obtained
from the initial 400 bp RT-PCR clones (Appendices F.2 and G.4) and expected to
produce a 206 bp product. Total RNA (ca. 3 μg) was used for the first strand cDNA
synthesis using 3' primers and reverse transcriptase (Superscript, Gibco-BRL) with
each of the 0, 5, 10, 20 or 30 dap as well as with seedling tissue and floral apical
meristem RNA extracts (positive controls), following the manufacturer's directions
from the 5' RACE kit (Gibco-BRL). The protocol was modified in the first strand
synthesis where the 3' primer for actin was used in the same RT reaction as the 3'
primer for DFR so as to produce two cDNAs in one reaction from the same aliquot of
RNA per seed stage. In this way, the generation of cDNA from the set of RNA
samples of developing seed was quantified by synthesis of a constitutive transcript
according to recognized protocols (Yu et al., 1998; Ride et al., 1999; Shimizu et al.,
1999; Mato et al., 2000). The subsequent PCR reactions were performed separately
using either primer set for these combined cDNA templates.

Amplification by PCR (Appendix F.3) was expected to generate a ca. 650 bp
fragment from the actin cDNA or a ca. 206 bp fragment from the DFR cDNA. The
number of program cycles required for logarithmic PCR-amplification of DFR
transcript in developing seed was determined by increasing the number of cycles by
two between 28 and 40 cycles. Optimal primer concentrations (either 0.4 or 0.6 μM)
were tested for both actin and DFR. Individual control PCR assays were used that
excluded either the 5' primers, the 3' primers, or the cDNA template mixture.
CHAPTER 4

RESULTS

4.1 Seed coat pigmentation in the Brassicaceae

Genetically-related, yellow- and brown-seeded lines of *B. carinata* were developed from yellow-seeded PGRC/E 21164 germplasm (Figure 3.1, section 3.1). Of the initial 56 plants, 43 produced seed with unpigmented seed coats and 13 produced seed with pigmented seed coats. Since the original seed came from a population of unknown pedigree, these numbers cannot be interpreted as a meaningful ratio. No brown seed developed on any of the S2 and S3 plants grown from the yellow-seeded progeny used to produce the Y line, although in the cooler growing conditions for S2 plants, the ripened seed was a darker yellow compared to seed from plants in the warmer conditions. There was pigmentation around the hilum and scattered in small spots over the seed coat in all yellow-seeded samples (Figure 4.1, A). Plants in the B line produced uniformly dark brown seeds with no detectable pigment concentrated in spots (Figure 4.1, C). In contrast, the seed of *B. carinata* cv S-67 had a lighter brown seed colour than the B line (Figure 4.1, B) and in its overall appearance, *E. sativa* had black seed (Figure 4.1, D).

Seed from the plants of Y and B lines grown in the field was pigmented or unpigmented to the same degree as the seed planted in the growth chamber. This confirmed that the seed coat pigmentation trait was stable under field conditions.
Figure 4.1  Mature whole seeds of *Brassica carinata* and *Eruca sativa*
illustrating the variation in the seed coat pigmentation
character. Seeds were imbibed in water for 3 h in order to
reveal pigmentation that is less evident in dry seed material.
(A) Line PGRC/E 21164 Y: arrows indicate pigmented hilum;
spotty, scattered pigmentation evident throughout the seed.
(B) cv S67: brown to light brown seed harvested from one
siliqua; degree of pigmentation was not uniform.
(C) Line PGRC/E 21164 B: uniformly dark brown seed.
(D) *E. sativa* SRS 2681 dry seed appears black; imbibed seed
appears purple-black.
4.2 Identification of *in situ* seed coat pigments by histological techniques

Prior to staining the seed coat, fresh seed coat material from dark- or brown-seeded Brassicaceae samples was a uniform brown colour with a dark area around the hilum and some darker spots or mottling when viewed under the microscope (*B. carinata* only shown, Figure 4.2, D and G). The black-seeded *Eruca sativa* SRS 2681 (Figure 4.1, D) was unusual. When removed from the embryo, this seed coat was actually light-brown (Figure 4.2, J) and the purple-black embryonic cotyledons imparted the overall black appearance to the seed. The cotyledons instantly turned bright red when BuOH-HCl was added, an indication that anthocyanins were present. The composition of this pigment was not investigated further. Embryonic tissue from the other specimens was not coloured in this way.

The acidified solvent treatments (EtOH-, MeOH-, BuOH-, vanillin-HCl) did not appear to cause an immediate colour reaction in any of the dissected seed coats, indicating that anthocyanin was not present in this tissue of these species of Brassicaceae (Table 4.1). Over the next 2 to 6 h, a bright red colour developed in many of the dark-seeded specimens and around the hilum in some of the yellow-seeded material (Figure 4.2, C). Vanillin-HCl treatment was more effective in staining seed coat tissue bright red when preceded by a 2:1 (v/v) EtOH-HCl treatment for 30 min. Due to their very small size, seeds from *A. thaliana* were monitored by microscope to verify that no anthocyanin was present. The only changes in colour were the same as those observed for the dark-seeded specimens of other Brassicaceae and there was no evidence of anthocyanins in *Arabidopsis* seed. After 24 h, the bright red staining deepened to a burgundy red, indicative of the presence of condensed tannins. The
Figure 4.2  Dissected seed coat of *Brassica carinata* and *Eruca sativa* with histological stains or wet mounted.

Magnification: A to J, 62.5x; K and L, 25x.

Bar, lower left of each frame, 100 μm.


B, E, H, K: histochemically treated with vanillin-HCl (≥24 h); (view of seed coat not including hilum area.)

C, F, I, L: histochemically treated with vanillin-HCl (≥24 h); (view of seed coat, white arrow showing the hilum).

❖ *B. carinata*, Y line (A to C): in (B), ϕ indicates brown pigment that does not change with any histochemical treatment described.

❖ *B. carinata*, B line: D to F.

❖ *B. carinata*, cv S-67: G to I.

❖ *Eruca sativa*, SRS 2681 (J to L): no red coloured area in seed coat.
### Table 4.1 Analysis of pigments in seed coat tissue from selected Brassicaceae.

**Presence of CT\(^1\) based on development of red colour with histochemicals**

<table>
<thead>
<tr>
<th>Genus/species</th>
<th>Seed colour</th>
<th>Anthocyanins</th>
<th>CT (hilum)</th>
<th>CT (entire)</th>
<th>CT (spots(^2))</th>
<th>Brown pigment(^3)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. carinata</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S-67, field-grown</td>
<td>brown</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>n.d.(^4)</td>
<td>+</td>
</tr>
<tr>
<td>(S_2) generation</td>
<td>yellow</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>(S_2) (B line)</td>
<td>dark-brown</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>n.d.(^4)</td>
<td>+</td>
</tr>
<tr>
<td>(S_3) (Y line)</td>
<td>yellow</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>B. juncea</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Common Brown</td>
<td>brown</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>n.d.(^4)</td>
<td>-</td>
</tr>
<tr>
<td>AC Vulcan</td>
<td>yellow</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>variable</td>
<td>+</td>
</tr>
<tr>
<td>Lethbridge 22A</td>
<td>yellow</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
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<tr>
<td><em>B. rapa</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Echo</td>
<td>purple-black</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>n.d.(^4)</td>
<td>n.d.(^4)</td>
</tr>
<tr>
<td>AC Parkland</td>
<td>yellow</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
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<tr>
<td><em>B. napus</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>YN90-1016 (parent 1)</td>
<td>yellow</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+(^2)</td>
<td>-</td>
</tr>
<tr>
<td>KF95-5D6*</td>
<td>yellow</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+(^2)</td>
<td>-</td>
</tr>
<tr>
<td>KF95-1A5*</td>
<td>yellow</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+(^2)</td>
</tr>
<tr>
<td>KF95-4E4*</td>
<td>yellow</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+(^2)</td>
</tr>
<tr>
<td>KF95-5C8*</td>
<td>brown</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>variable</td>
<td>variable</td>
</tr>
<tr>
<td>Apollo (parent 2)</td>
<td>dark brown</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>n.d.(^4)</td>
<td>n.d.(^4)</td>
</tr>
<tr>
<td>Westar</td>
<td>dark brown</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>n.d.(^4)</td>
<td>+</td>
</tr>
<tr>
<td>*B. nigra SRS(^5) 192</td>
<td>brown</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>n.d.(^4)</td>
<td>-</td>
</tr>
<tr>
<td><em>B. oleracea</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SRS 652</td>
<td>brown</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>n.d.(^4)</td>
<td>-</td>
</tr>
<tr>
<td>SRS 2400</td>
<td>brown</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>variable</td>
<td>variable</td>
</tr>
<tr>
<td><em>Sinapis</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. alba</em> cv Ochre</td>
<td>pale yellow</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>S. alba</em> SRS 2495</td>
<td>brown</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td><em>S. arvensis</em></td>
<td>black</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td><em>Eruca sativa</em> SRS 2681</td>
<td>Lt.(^6) brown</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td><em>Raphanus sativus</em></td>
<td>pink-brown</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Crambe abyssinica</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(cv Prophet)</td>
<td>black</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td><em>A. thaliana</em></td>
<td>brown</td>
<td>-</td>
<td>n.d.(^4)</td>
<td>-</td>
<td>-</td>
<td>n.d.(^4)</td>
</tr>
</tbody>
</table>

\(^1\) condensed tannin (slow colour formation; unextractable); \(^2\) includes mottled patches; \(^3\) no colour change (unextractable); \(^4\) not detectable; \(^5\) Saskatoon Research Station; \(^6\) light; \(^*\) F\(_5\) progeny of YN1016 x Apollo

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BuOH-HCl reagent formed a burgundy red-coloured product within 24 h; vanillin-HCl was slower to stain the seed coats red but produced the brightest red colour. By 24 h, most specimens displayed deep red staining around the hilum area with the histochemicals, regardless of the overall seed colour (Table 4.1). In the black- or brown-seeded cultivars (B. carinata, B line, cv S-67; B. juncea ‘Common Brown’; B. rapa cv Echo; B. napus, cv Apollo, cv Westar; B. oleracea SRS 652, SRS 2400; S. alba SRS 2495, S. arvensis; R. sativus, A. thaliana), seed coats were stained red throughout the entire seed coat, with deeper red colour at the hilum area (Table 4.1; Figure 4.2, C, F, I). The brown-seeded specimens, C. abyssinica and E. sativa, were exceptions because these seed coats did not stain red at either the hilum or in any other areas (Figure 4.2, K, L). Even after 96 h immersion in histochemical stain, these seed coats appeared uncoloured (or blue-gray, possibly due to cell wall-refracted light) (Figure 4.2; K). In the hilum area of E. sativa and C. abyssinica, the tissue also remained a dark brown colour after 96 h of treatment (Figure 4.2, L).

In some samples, the histochemical treatment stained scattered spots or large patches throughout the seed coat a very deep red, indicative of a concentrated area of condensed tannin (Figure 4.2, F). In very dark-seeded material (B. carinata B line; B. juncea ‘common brown’; B. napus cv Apollo and cv Westar; B. oleracea SRS 652), the overall red staining was too deeply coloured to detect any concentrated spots that may have been present in the dark red background. This was indicated in Table 4.1 as ‘not detectable’ (n.d.). Brown-seeded B. napus KF95-5C8 and B. oleracea SRS 2400 were variable, in that some seed coats were stained with a spotty red pattern against the red stained background, while other seed coat samples were completely devoid of the very dark red spots. This observation prevailed in repeated experiments. Brassica oleracea
SRS 2400 may originally be from a population that does not uniformly express this patterning trait.

In several brown-seeded specimens (*B. carinata* cv S-67 and the B line; *S. alba* SRS 2495, *S. arvensis*), a brown pigment that did not change colour with any of the histochemicals was present in scattered spots in the seed coat (Figure 4.2, B; Table 4.1). This brown colour was evident even when most of the seed coat stained red with the histochemical treatments. The appearance of this unextractable pigment resembled the description of phlobaphenes in maize pericarp by Styles and Ceska (1989) and Grotewold *et al.* (1994). In some of the deeply-stained seed coat of very dark-seeded samples (*B. rapa* cv Echo; *B. napus* cv Apollo and cv Westar and *A. thaliana*) unchanged brown pigment that may have been present as small spots or patches in the dark-red background was difficult to detect. This is recorded as ‘n.d.’ in the column for brown pigment (Table 4.1).

In two yellow-seeded specimens (*B. napus* YN90-1016, KF95-5D6), only the hilum and the scattered spots changed to deep-red with histochemical treatments. indicative of condensed tannins. No additional brown pigmentation was evident. unlike a related brown-seeded *B. napus* KF95-5C8, one of the progeny from the same cross as KF95-5D6 (see Table 4.1). These latter seeds were very mottled and stained red in some places or remained brown (unchanged) in other patches.

In other yellow-seeded specimens, there were no detectable colour changes due to any of the histochemical treatments: yellow-seeded cultivars of *B. juncea* cv Lethbridge 22A, *B. rapa* cv AC Parkland and *S. alba* cv Ochre did not stain red in the hilum area and showed no red or burgundy-red colour elsewhere. In addition, these yellow-seeded specimens (except *S. alba* cv Ochre) displayed a brown pigment in the
hilum area that did not change colour over the course of the 96 h histochemical treatment.

Brown pigment that did not stain red was also present in scattered spots in seed coats of some yellow-seeded material (B. carinata, S2 and Y line [indicated by ‘p’, Figure 4.2, B]; B. juncea cv AC Vulcan) even when a few isolated spots stained red. In some accessions (e.g. yellow-seeded B. napus [progeny KF95-1A5 and KF95-4E4]), this brown pigment was present in large patches in the seed coats.

Embryonic tissue was examined in Eruca sativa SRS 2681. This dark-seeded species differed from all of the other Brassicaceae tested because the isolated seed coat was light brown and the embryo was purple-black, thereby imparting an overall dark appearance to the intact seed. When the isolated seed coat was flooded with vanillin-HCl or BuOH-HCl, there were no colour changes even after 5 d in the histochemicals. The embryo immediately turned bright pink-red when treated with an acidic solvent, which was indicative of anthocyanin pigment.

4.3 Quantitation of condensed tannin in seeds of the Brassicaceae

The PVPP-BuOH-HCl assay for extractable condensed tannins from whole seeds detected condensed tannins in whole seed samples. However, the ground seed was still very pigmented after extraction in BuOH-HCl. A second experiment to extract pigment from isolated seed coats by heating in BuOH-HCl for 1 h at 70°C did not displace the pigment. There was an obvious difference in the amount of extractable condensed tannin between some of the yellow- and brown-seeded material (Figure 4.3), but since most of the pigment appeared to remain trapped in the seed coat, these values do not represent an accurate estimate of the concentration of condensed tannins.
Figure 4.3 Concentration of extractable condensed tannin in whole seed material.

The BuOH-HCl assay (Skadhauge et al., 1997) to estimate condensed tannins was used with bulked samples of seed from yellow- and brown-seeded accessions of selected Brassicaceae.

Legend: open bars, yellow-seeded samples; striped bars, brown- or black-seeded samples. Error bars represent SE. *No extractable condensed tannin detected; SRS, Saskatoon Research Centre accession number; com. brn, common brown; g.c., growth chamber.
4.4 Anthocyanin in seeds and leaf tissue from *B. carinata*

4.4.1 Leaf colour differences between *B. carinata* Y and B line plants

There was a distinctive difference in the leaf colour of young seedlings at the two- to four-leaf stage of *B. carinata* Y line compared to the genetically-related B line. The leaves on plants from the Y line were lighter and lacked the dark green-blue tinge evident in leaves from the B line (Figure 4.4). This difference in pigmentation applied only to true leaves. Young cotyledons of both lines appeared to contain anthocyanins, regardless of the temperature. After the five-leaf stage, seedlings grown from the Y line acquired the darker bluish-green leaf colour comparable to the B line plants. If plants were not grown at warm daytime temperatures (20 to 25°C) or were chilled, the leaves from yellow-seeded material rapidly became the same colour as seedlings grown from brown-seeded material. It was later noted that if the developing plants remained under cooler temperatures (15 to 18°C) in a greenhouse, mature seed from Y line material was a brownish-yellow, whereas, plants grown under warm temperatures (ca. 20 to 25°C) produced seed that was yellow.

4.4.2 Anthocyanins from seed and leaf tissue of *B. carinata*

Since anthocyanins had not been detected in individual, isolated seed coats of *B. carinata* by histochemical techniques (section 4.2), 2-g seed material was extracted for anthocyanin testing (section 3.6). Analysis of the *iso*-amyl alcohol extracts from both Y and B seeds by TLC did not show any anthocyanin pigments. The leaves on plants grown (20 to 25°C) from yellow-seeded material contained only trace amounts of anthocyanins that were faintly visible on a 1D-TLC plate. In contrast, the leaf tissue extracts from plants grown from brown-seeded material contained enough pigment to be distinctly visible in solution (Figure 4.5).
Figure 4.4  *Brassica carinata* PGRC/E 21164 Y and B line seedlings at the four leaf stage, grown at 20 to 25°C. (A) B line  (B) Y line

Figure 4.5  Comparison of *iso-amyl* alcohol extracts of leaf tissue after 2 M HCl hydrolysis. (A1) Anthocyanins from B line leaf tissue  (B1) Absence of anthocyanins from Y leaf tissue.
Two anthocyanin-like compounds could be detected after separation of B line leaf extracts on 1D-TLC cellulose plates. Both pigmented compounds had a pink-orange colour. The colour and separation characteristics (Forestal, \( R_f \) 67, 77; BAW, \( R_f \) 51, 61) were similar to those of pelargonidin, but neither compound co-migrated exactly with the authentic standard (Forestal, \( R_f \) 69; BAW, \( R_f \) 55). The wavelength scan of the crude iso-amyl alcohol leaf extract before 1D-TLC separation produced a very complex absorbance pattern, with a maximum absorbance at 220 nm, one broad absorbance peak at 300 nm and a small peak at 550 nm, where anthocyanins might be expected to absorb (data not shown). The concentration of anthocyanin purified by elution from the 2D-TLC was too low for detection by a wavelength scan.

4.5 Thioglycolic lignin in selected Brassicaceae

4.5.1 Method development in the thioglycolic lignin assay

The thioglycolate lignin assay relies on the removal of all non-lignin material absorbing in the 280 nm region so that the absorbance of the final thioglycolic lignin preparation is due only to the lignin. Certain compounds have been suspected to exaggerate thioglycolic lignin estimates because monolignols and other phenolics can form many types of cross-linkages with cell walls and proteins (Whitmore, 1978a). Phenolic esters (e.g. sinapine) are abundant seed components in the Brassicaceae and these have absorption maxima in the 280 nm region. It is therefore important that these compounds be removed from extracts used for thioglycolic lignin estimates (section 3.7; Appendix B.2.2).

The thioglycolic lignin extraction was tested for its efficiency in removing protein or sinapine, both of which would interfere with absorbance readings \( (\lambda_{280}) \). The control samples of sinapine and the two types of protein (gelatin and bovine serum
albumin) produced no pellet at the acid precipitation step and thus no absorbance at 280 nm. This established that free sinapine or protein alone did not carry through the extraction to influence the final absorbance values. To measure whether hydroxyproline-rich proteins formed a derivative with gelatin and the lignin (test sample F) were combined and analyzed. The absorbance ($\lambda_{280}$) was 0.502 AU which was similar to the absorbance in the lignin-only sample (0.521 AU).

In a separate experiment, the incubation time for thioacetolysis was varied to ensure that 3 h was sufficient for consistent derivatization and consequently, consistent absorbance measurements. Samples were reacted with thioglycolic acid for a total of 4 h, with subsamples removed every 30 min. The subsample assays were completed according to the thioglycolic lignin protocol. There was a linear relationship between time and absorbance (1 h to 3 h). After this time, the absorbance began to decrease compared to the 3 h samples and the lignin values in the replicate samples varied widely when sampled later than 3 h. The MeOH wash step was improved by using 80% MeOH and washing the ground whole seed twice, once with 80% MeOH and once with 100% MeOH. Sinapine was removed more effectively with 80% MeOH than with 100% MeOH because the absorbance ($\lambda_{280}$) of the wash solution dropped to nearly zero on the second wash when 80% MeOH was used first. Three or more washes were required to reduce the absorbance to nearly zero when only 100% MeOH was used.

Based on these methods, it was found that lignin concentration was significantly lower in cultivars and germplasm of yellow-seeded Brassicaceae (6.14 $\mu$g mg$^{-1}$ whole
seed; n = 12) compared to the brown-seeded Brassicaceae (13.99 \( \mu \text{g} \ \text{mg}^{-1} \) whole seed; n = 18) (t = 6.283; p = 0.0000) (Figure 4.6; Table 4.2 A).

4.5.2 Thioglycolic lignin concentration in seed coat and embryo tissue

Lignin concentration was examined in dissected seed coats and embryos from the three generations of *B. carinata* that contributed to the development of the *B. carinata* Y line and the two generations that contributed to the development of the *B. carinata* B line. Lignin concentration was significantly lower in seed coat tissue of yellow-seeded *B. carinata*, averaged over all three generations (99.01 \( \mu \text{g} \ \text{mg}^{-1} \) seed coat; n = 35), than in the brown-seeded material, averaged over the two generations (293.71 \( \mu \text{g} \ \text{mg}^{-1} \) seed coat; n = 29) (Figure 4.7 A; Table 4.2A).

In embryo tissue, lignin concentration averaged 22.16 \( \mu \text{g} \ \text{mg}^{-1} \) embryo tissue in the yellow-seeded material (n = 35) and 17.32 \( \mu \text{g} \ \text{mg}^{-1} \) embryo in the brown-seeded material (n = 29) which was not significantly different (Table 4.2A, Figure 4.7 B). Dissected seed coat tissue was weighed for the thioglycolic lignin determinations and it was found that the seed coat of the Y line weighed significantly less compared to the B line of *B. carinata* (136.5 and 163.1 mg g\(^{-1}\) whole seed, respectively) (Table 4.2B).

4.6 Characterization of colourless phenolics in *B. carinata*

4.6.1 Evaluation of MeOH extraction procedures

Several concentrations of MeOH were tested for their efficacy in extracting phenolic compounds from seeds (section 3.8). The most effective extraction for *Brassica* seed was achieved with 80% MeOH. The extraction procedure was evaluated on the basis of the amount of fluorescence for each spot that was detectable under UV-illumination after 1D-TLC separation. Two large fluorescent-blue spots were observed.
Figure 4.6  Concentration of lignin in whole seed material from brown- and yellow-seeded samples of the Brassicaceae.

Lignin concentration in brown-seeded material (striped bars); lignin concentration in yellow-seeded material (open bars). Error bars obscured in some samples due to the very low SE.

(Abbreviations: Com. Br., common brown; f.g., field grown; g.c., growth chamber grown; SRS, Saskatoon Research Centre accession number.)
Table 4.2A Comparison of thioglycolic lignin concentration among yellow- and brown-seeded Brassicaceae and two types of yellow- and brown-seeded Brassica carinata tissues, using Student’s t test.

<table>
<thead>
<tr>
<th>Sample</th>
<th>n(^1)</th>
<th>mean lignin (µg mg(^{-1}) tissue)</th>
<th>(t_{(0.005)})</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Whole seed samples</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yellow-seeded Brassicaceae</td>
<td>12</td>
<td>6.14</td>
<td>6.283</td>
<td>0.0000**</td>
</tr>
<tr>
<td>Brown-seeded Brassicaceae</td>
<td>18</td>
<td>13.99</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Dissected seed coat samples</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yellow-seeded B. carinata</td>
<td>35</td>
<td>99.01</td>
<td>11.974</td>
<td>0.0000**</td>
</tr>
<tr>
<td>Brown-seeded B. carinata</td>
<td>29</td>
<td>293.71</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Dissected embryo samples</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yellow-seeded B. carinata</td>
<td>35</td>
<td>22.16</td>
<td>2.071</td>
<td>0.0426 NS</td>
</tr>
<tr>
<td>Brown-seeded B. carinata</td>
<td>29</td>
<td>17.32</td>
<td></td>
<td></td>
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</tbody>
</table>

Table 4.2B  Comparison of weight of dissected seed coat from S\(_1\) to S\(_3\) generations of yellow- and S\(_1\) to S\(_2\) generations of brown-seeded Brassica carinata

<table>
<thead>
<tr>
<th>Sample</th>
<th>n(^1)</th>
<th>mean weight (mg g(^{-1})seed coat)</th>
<th>(t_{(0.01)})</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yellow-seeded generations</td>
<td>36</td>
<td>136.5</td>
<td>5.640</td>
<td>0.000*</td>
</tr>
<tr>
<td>Brown-seeded generations</td>
<td>29</td>
<td>163.1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(n\(^1\),\ number\ of\ observations\)

**highly significant difference; *significant; NS, not significant

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Figure 4.7  Thioglycolic lignin concentration in dissected seed coat and embryo tissue of *Brassica carinata* PGRC/E 21164 Y and B lines. Error bars represent SE.
on cellulose TLC plates developed with 15% HOAc from 20-μL samples of the 50% and 60% MeOH extracts. These blue spots were identified as sinapine and sinapic acid (Rt 74 and 40, respectively). The 80% and 100% MeOH extracts contained several other discrete spots (e.g. unconfirmed flavonols that fluoresced orange-brown at, or near, the origin under UV-illumination in the yellow-seed extract only). The fluorescent-blue spot sizes were larger in the 80% MeOH extract and there appeared to be less streaking at the solvent front compared to the 100% MeOH extract. On the basis of these observations, 80% MeOH was chosen as the extraction solvent.

4.6.2 Characterization of seed coat extracts by 2D-TLC

The 2D-TLC separations revealed some distinctive phytochemical differences between hand-dissected yellow and brown seed coat tissue of B. carinata. Compounds in these seed coat extracts maintained the tightest spot configuration with 30% HOAc as the first dimension solvent. In addition, phenolic acids and their esters were resolved from the flavonol and dihydroflavonol classes of compounds in 30% HOAc (Appendix H). Forestal was the best mobile phase for the second dimension in these experiments. Compounds that did not migrate very far from the origin in 30% acetic acid could be resolved in Forestal without migrating closely together near the solvent front, as occurred when BAW was used as the second mobile phase (Appendix H.2).

Extracts of Y line B. carinata seed coats contained an abundance of phenylpropanoid and flavonoid compounds, unlike the extracts of B line or cv S-67 (Figures 4.8, 4.9, 4.10). In the Y line extract, t-cinnamic acid and the dihydroflavonols (dihydrokaempferol, dihydroquercetin and dihydromyricetin) appeared to be present (Figure 4.8, spot nos. 2-5). Preliminary identification was based on colour under UV-
Figure 4.8  Two dimensional separation pattern of *Brassica carinata* Y line seed coat extracts (from hand-dissected seed) on cellulose TLC developed with 30% HOAc (first dimension) and Forestal (second dimension). Viewed by UV-illumination ($\lambda_{366}$), shown without NH$_3$ fuming. Spot colour rather than intensity is representative of actual appearance.

Legend:
- '●', origin of sample; '●', origin of $t$-cinnamic acid standard developed in the first dimension with 30% HOAc; '●', origin of $t$-cinnamic acid standard developed in the second dimension with Forestal.

Identified compounds:
1. similar to sinapic acid, (UV-spectrum, see section 4.6.4)
2. cross-hatched spot, $t$-cinnamic acid, visible only under UV $\lambda_{280}$
3. dihydrokaempferol
4. dihydroquercetin
5. dihydromyricetin
Compounds 6, 7, 8 unidentified (present in seed coat extracts of both the Y and B lines).
Dashed line spot, not visible until after NH$_3$ fuming.
Figure 4.9  Two dimensional separation pattern of *B. carinata* B line seed coat extracts (from hand-dissected seed) on cellulose TLC developed with 30% HOAc (first dimension) and Forestal (second dimension). Viewed by UV-illumination (λ<sub>366</sub>), shown without NH₃ fuming.

**Legend:**
- †, origin of sample; ‡, origin of dihydrokaempferol standard developed in the first dimension with 30% HOAc; ‡, origin of dihydrokaempferol standard developed in the second dimension with Forestal. Compounds 6, 7, 8 present in seed coat extracts of both the Y and B lines.
Figure 4.10  Two dimensional separation pattern of *Brassica carinata* cv S-67 seed coat extracts (from hand-dissected seed) on cellulose TLC developed with 30% HOAc (first dimension) and Forestal (second dimension). Viewed by UV-illumination ($\lambda_{366}$), shown without NH$_3$ fuming. Spot colour rather than intensity is representative of actual appearance.

Legend:
'●', origin of sample; '●', origin of eriodictyol standard developed in the first dimension with 30% HOAc; '●', origin of eriodictyol standard developed in the second dimension with Forestal.
(9) compound similar to eriodictyol (flavanone); (10) dashed line spot, visible only after fuming with NH$_3$. 

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illumination (with and without NH₃) and their co-migration with authentic standards in the second dimension. The compounds in the Y line extract (spot nos. 1-5, Figure 4.8) were absent from the extracts prepared from brown-seeded material. The B line seed coat extract had the least number of total phenolic components of all three lines (Figure 4.9). Characteristics of the compounds (with and without NH₃ fuming) on the three 2D-TLC plates are summarized in Table 4.3. Some of these compounds did not appear to correlate with the seed coat pigmentation trait and further investigation into their identities was not undertaken.

In separate TLC experiments (Appendix H.2), sinapic acid and sinapine were compared to the unknown phenolic that was detected only in Y line extracts (spot no. 1, Figure 4.8). Although sinapic acid and the unknown were very similar in colour under UV-illumination, their migration characteristics on TLC were dissimilar (Rₐ 91 of the unknown in Forestal, compared to Rₐ 82 for sinapic acid and Rₐ 96 for sinapine). It is possible that the unknown compound was an esterified form of sinapic acid (A. Muir, pers. comm.). No sinapine, flavanones or free phenolic acids (other than the phenylpropanoid, t-cinnamic acid) were detected by 2D-TLC in hand-dissected seed coat extracts of the B. carinata Y line. The characteristic mobility of spots no. 7 and 8 (Figures 4.8, 4.9; very low Rₐ value in 30% HOAc and very high Rₐ value in Forestal) is typical of flavonols (Table H.2, Appendix).

4.6.3 Characterization by RP-HPLC of unhydrolyzed MeOH extracts

Samples of phenolics from whole seeds of Brassica species were collected by MeOH extraction (section 3.10.1). Seed material represented different cultivars and germplasm from the six Brassica species described by U (1935) (Table 4.4 and
Table 4.3  Summary of compounds identified in the seed coat extracts of *Brassica carinata* Y and B lines and cv S-67 from cellulose 2D-TLC.

<table>
<thead>
<tr>
<th>Spot No.</th>
<th>Colour under UV λ&lt;sub&gt;366&lt;/sub&gt;</th>
<th>Colour with NH&lt;sub&gt;3&lt;/sub&gt;</th>
<th>other characteristics</th>
<th>preliminary identification</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>blue-green</td>
<td>turquoise</td>
<td>similar colour to sinapic acid; higher R&lt;sub&gt;f&lt;/sub&gt; value&lt;sup&gt;*&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>invisible</td>
<td>invisible</td>
<td>black under λ&lt;sub&gt;280&lt;/sub&gt;</td>
<td>t-cinnamic acid</td>
</tr>
<tr>
<td>3</td>
<td>purple-black</td>
<td>no change</td>
<td>dihydrokaempferol</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>purple-black</td>
<td>no change</td>
<td>dihydroquercetin</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>purple-black</td>
<td>no change</td>
<td>dihydromyricetin</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>pale yellow</td>
<td>brightens</td>
<td>flavone-like in mobility</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>orange</td>
<td>brightens</td>
<td>flavonol-like in mobility</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>deep yellow</td>
<td>brightens</td>
<td>flavonol-like in mobility</td>
<td></td>
</tr>
</tbody>
</table>

*Y line extract (2D-TLC, Figure 4.8)*

<table>
<thead>
<tr>
<th>Spot No.</th>
<th>Colour under UV λ&lt;sub&gt;366&lt;/sub&gt;</th>
<th>Colour with NH&lt;sub&gt;3&lt;/sub&gt;</th>
<th>other characteristics</th>
<th>preliminary identification</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>pale yellow</td>
<td>brightens</td>
<td>identical in behaviour and mobility to Y line 6</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>orange</td>
<td>brightens</td>
<td>identical in behaviour and mobility to Y line 7</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>deep yellow</td>
<td>brightens</td>
<td>identical in behaviour and mobility to Y line 8</td>
<td></td>
</tr>
</tbody>
</table>

*B line extract (2D-TLC, Figure 4.9)*

<table>
<thead>
<tr>
<th>Spot No.</th>
<th>Colour under UV λ&lt;sub&gt;366&lt;/sub&gt;</th>
<th>Colour with NH&lt;sub&gt;3&lt;/sub&gt;</th>
<th>other characteristics</th>
<th>preliminary identification</th>
</tr>
</thead>
<tbody>
<tr>
<td>9</td>
<td>orange</td>
<td>intensifies</td>
<td>similar to eriodictyol</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>invisible</td>
<td>dark blue</td>
<td>visible only after fuming with NH&lt;sub&gt;3&lt;/sub&gt;</td>
<td></td>
</tr>
</tbody>
</table>

* discussed further in the text
Table 4.4  Phenolic acid profile\(^1\) of *Brassica* species representing the diploid and amphidiploid genomes of the Triangle of U.

<table>
<thead>
<tr>
<th>Genus/species Cultivar/Accession No.</th>
<th>Genome</th>
<th>Seed colour</th>
<th>RT range (min)(^2) (simple phenolic acids)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Brassica carinata</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PGRC/E 21164 ((S_1))</td>
<td>BB CC</td>
<td>yellow</td>
<td>absent</td>
</tr>
<tr>
<td>cv S-67</td>
<td></td>
<td>brown</td>
<td>absent</td>
</tr>
<tr>
<td><em>Brassica napus</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>YN 90 (parent 1)</td>
<td>AA CC</td>
<td>mottled yellow</td>
<td>7.2–7.4</td>
</tr>
<tr>
<td>KF95 5D6 (progeny)</td>
<td></td>
<td>patchy brown/yellow</td>
<td>7.2–7.4</td>
</tr>
<tr>
<td>KF95 1A5 (progeny)</td>
<td></td>
<td>patchy brown/yellow</td>
<td>7.0–7.4</td>
</tr>
<tr>
<td>KF95 4E4 (progeny)</td>
<td></td>
<td>patchy brown/yellow</td>
<td>7.2–7.4</td>
</tr>
<tr>
<td>KF95 5C8 (progeny)</td>
<td></td>
<td>brown</td>
<td>7.2–7.4</td>
</tr>
<tr>
<td>cv Apollo (parent 2)</td>
<td></td>
<td>dark brown</td>
<td>7.2–7.4</td>
</tr>
<tr>
<td>cv Westar</td>
<td></td>
<td>brown</td>
<td>6.0–7.4</td>
</tr>
<tr>
<td><em>Brassica juncea</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ZYR-6</td>
<td>AA BB</td>
<td>yellow</td>
<td>7.2–7.4</td>
</tr>
<tr>
<td>cv Lethbridge 22A</td>
<td></td>
<td>yellow</td>
<td>7.2–7.2</td>
</tr>
<tr>
<td>cv Blaze</td>
<td></td>
<td>brown</td>
<td>7.2–7.4</td>
</tr>
<tr>
<td>common brown</td>
<td></td>
<td>brown</td>
<td>7.2–7.4</td>
</tr>
<tr>
<td><em>Brassica rapa</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cv AC Parkland</td>
<td>AA</td>
<td>yellow</td>
<td>7.2–7.4</td>
</tr>
<tr>
<td>cv Echo</td>
<td></td>
<td>purple-black</td>
<td>6.0–7.4</td>
</tr>
<tr>
<td><em>Brassica nigra</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cv Bari</td>
<td>BB</td>
<td>brown</td>
<td>absent</td>
</tr>
<tr>
<td>SRS 192</td>
<td></td>
<td>brown</td>
<td>absent</td>
</tr>
<tr>
<td>SRS 652</td>
<td></td>
<td>brown</td>
<td>absent</td>
</tr>
<tr>
<td>SRS 2400</td>
<td></td>
<td>brown</td>
<td>absent</td>
</tr>
</tbody>
</table>

\(^{1}\)Separation by RP-HPLC (system 'A') using a LiChrospher C\(_{18}\) column and a 55-min gradient (section 3.10.1).

\(^{2}\)RT, retention time. Here, RT is presented as a range (min) to designate the group of simple phenolic acids (having >20 mAU at 330 nm) that occur in the chromatograms of the A genome extracts.
appendix A). These samples, analyzed by HPLC 'A' (section 3.3.1) contained mainly
sinapine (ca. RT 11.0) and unidentified phenolic acids (ca. RT 5.9 to 7.5 min) (Table
4.4; Figure 4.11). In species without the A genome (B. carinata, B. nigra, B. oleracea),
there were no detectable phenolic acids until sinapic acid eluted (RT > 9.0 min). A
characteristic group of phenolic acids were present in the A genome samples only and
eluted between 5.9 and 7.4 min (Figure 4.11). Two of the detected peaks (ca. RT 7.2
and 7.4) occurring in this group had UV-spectra that were nearly identical. The elution
pattern of these phenolic acids within each species was very similar or identical.
Differences in phenolic acids that related to whether the Brassica sample was diploid
(AA) or amphidiploid (AACC, AABB) were not confirmed, although the samples of B.
rapa (diploid) tested for this experiment appeared to contain lower amounts of phenolic
acids eluting between 7.1 and 7.4 min than did B. napus (amphidiploid) or B. juncea
(amphidiploid). According to the UV-spectral library, simple phenolic acids possess
RTs and UV-spectra similar to the components identified in these whole seed extracts.
Concentration of the unknown phenolic acids in these extracts was too low (≤0.20 AU)
for exact identification by LC-MS-MS. Since these compounds did not have any
apparent relationship to seed colour traits (Table 4.4), identification of these phenolics
was not investigated further.

4.6.4 Compound identification by UV- and mass spectrometry

Methanolic B. carinata seed coat extracts (Y and B lines) were prepared from
mechanically-collected seed coat tissue. The preparations were subjected to acid
hydrolysis and aglycones were extracted with EtOAc. The EtOAc extract of Y line
/mechanically-collected seed coat) was further partitioned to yield three fractions
Figure 4.11 Phenolic acid profiles\(^1\) according to *Brassica* genomic class.

Chromatograms in figure are representative results of at least two injections for each of the accessions or cultivars shown:

(A) *Brassica nigra*, SRS 192 (Type 2)

(B) *Brassica carinata*, cv S-67

(C) *Brassica oleracea*, SRS 652

(D) *Brassica napus*, cv Westar

(E) *Brassica rapa*, cv AC Parkland

(F) *Brassica juncea*, SRS ZYR-6

\(^1\) Whole seed extracts (unhydrolyzed, 80% MeOH); seed colour reported in Table 4.4. Experiment was repeated three times.
(section 3.8). All extracts were analyzed by RP-HPLC (system ‘B’) and the spectrum of each peak compared to the UV-spectral library (Table H.5, Appendix H.3). The seed coat extracts were also analyzed by LC-MS-MS under soft ionization conditions (APCI) (system ‘C’, section 3.3.1).

The UV- and mass spectra confirmed the identification of compounds found by 2D-TLC (section 4.6.2) and UV-detection of RP-HPLC separated seed coat extracts from Y line samples. According to these analyses, there were small amounts of dihydromyricetin and dihydrokaempferol present. By comparison, the amount of dihydroquercetin was estimated at twice the concentration of dihydrokaempferol based on the area integrated under the peaks (Figure 4.12A). Compounds detected in Y line preparations and identified by their UV-spectra are shown in Table 4.5. Known compounds (e.g. l-cinnamic acid) were added as internal standards in Y and B line seed coat extracts to establish the alternate retention times that occur when compounds are resolved from complex mixtures (section 3.10.2). The increased size of the peak in the ‘spiked’ seed coat extract confirmed that l-cinnamic acid did have an increased retention time in the sample matrix of Y line seed coat extract (chromatogram of spiked analysis not shown).

The identification of l-cinnamic acid and the three dihydroflavonols by UV-detection using 2D-TLC (Table 4.3) and RP-HPLC (Table 4.5) separation methods was corroborated by single ion recording (SIR) of total ion current (TIC) scans (Table 4.6; Figures H.2-H.7, Appendix). The mass spectra were validated by authentic standards (Table 4.7) except for the methyl ester of sinapic acid (239 da). This compound was identified by (1) the UV-spectral match (nearly identical to sinapic acid, Table 4.5), (2)
Resolution of components in *Brassica carinata* Y and B line seed coat extracts by reversed-phase HPLC, system ‘B’, using a ‘Symmetry’ C\textsubscript{18} column, aqueous-acetonitrile gradient elution and PDA-detection*.

Compounds identified by comparison with RT and UV spectra of authentic standards (Table 4.5) and confirmed subsequently by LC-MS-MS (Tables 4.6, 4.7).

(A) Y line, 100% MeOH fraction: dihydrokaempferol and dihydromyricetin were present in trace amounts only compared to dihydroquercetin; flavanols (quercetin, kaempferol) and \( \tau \)-cinnamic acid were detected as shown; myricetin was not detected.

(B) B line, unFractionated extract: sinapic acid and sinapic acid methyl ester were the largest identified components present: compounds eluting at 3.9 and 11.0 min were absent after base hydrolysis. Subsequent to analytical HPLC, only trace amounts of quercetin and kaempferol were detected using mass spectrometry with SIR. The UV-spectrum of compound at 41.4 min is not similar to quercetin. No dihydroflavonols or \( \tau \)-cinnamic acid were detected by either RP-HPLC or LC-MS-MS.

*Chromatograms in figure are representative results of three or more injections. Selected standards were separated preceding and following extract injections as a check on the reproducibility of retention times.
A. 100% MeOH fraction of Y line extract

B. Unfractionated B line extract
Table 4.5  Compounds identified by UV-spectra and retention time in the seed coat extracts of Brassica carinata Y and B lines from RP-HPLC separations on a ‘Symmetry’ C\textsubscript{18} column (system ‘B’).

<table>
<thead>
<tr>
<th>RT (min)</th>
<th>Authentic</th>
<th>RT (min)</th>
<th>Match angle (degrees)</th>
<th>Absorption maxima\textsuperscript{1} (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unknown</td>
<td>Standard</td>
<td>Standard</td>
<td>Unknown (Standard)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Y line extract, 100% fraction, 55-min gradient (max plot, Figure 4.12, A)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14.5</td>
<td>dihydromyricetin</td>
<td>14.7</td>
<td>1.57</td>
<td>207, 295, 330\textsuperscript{ab2} (207, 292, 330\textsuperscript{b})</td>
</tr>
<tr>
<td>22.8</td>
<td>sinapic acid</td>
<td>23.2</td>
<td>1.53</td>
<td>200, 238, 324 (203, 239, 324)</td>
</tr>
<tr>
<td>24.8</td>
<td>dihydroquercetin</td>
<td>24.9</td>
<td>2.20</td>
<td>225\textsuperscript{ab}, 288, 330\textsuperscript{ab} (228\textsuperscript{ab}, 288, 330\textsuperscript{ab})</td>
</tr>
<tr>
<td>30.8</td>
<td>dihydrokaempferol</td>
<td>31.0</td>
<td>2.92</td>
<td>217, 288, 330\textsuperscript{ab} (216, 292, 330\textsuperscript{ab})</td>
</tr>
<tr>
<td>40.8</td>
<td>quercetin</td>
<td>40.1</td>
<td>0.65</td>
<td>203, 256, 369 (207, 256, 369)</td>
</tr>
<tr>
<td>44.5</td>
<td>\textit{t-}cinnamic acid</td>
<td>44.6</td>
<td>1.26</td>
<td>204, 277 (217, 279)</td>
</tr>
<tr>
<td>45.8</td>
<td>sinapic acid methyl ester</td>
<td>no standard available\textsuperscript{3}</td>
<td>238, 327</td>
<td></td>
</tr>
<tr>
<td>48.5</td>
<td>kaempferol</td>
<td>49.9</td>
<td>5.19</td>
<td>203, 256, 320\textsuperscript{ab}, 369 (206, 266, 320\textsuperscript{ab}, 366)</td>
</tr>
</tbody>
</table>

B line extract (total), 55-min gradient (max plot, Figure 4.12, B)

<table>
<thead>
<tr>
<th>RT (min)</th>
<th>Authentic</th>
<th>RT (min)</th>
<th>Match angle (degrees)</th>
<th>Absorption maxima (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>24.3</td>
<td>sinapic acid</td>
<td>23.2</td>
<td>1.34</td>
<td>203, 239, 324 (203, 239, 324)</td>
</tr>
<tr>
<td>47.4</td>
<td>sinapic acid methyl ester</td>
<td>no standard available</td>
<td>238, 327</td>
<td></td>
</tr>
</tbody>
</table>

\textsuperscript{1} in aqueous-acetonitrile-TFA gradient (HPLC system ‘B’)

\textsuperscript{2} (\textsuperscript{ab}) shoulder

\textsuperscript{3} UV-spectrum matched sinapic acid (match factor, 1.00)

\textit{note: the spectral bandwidth resolution of the PDA detector was 2.4 nm}
Table 4.6  Compounds identified by LC-MS-MS (RP-HPLC ‘C’) in extracts of (mechanically-collected) seed coats of Brassica carinata Y and B lines.

<table>
<thead>
<tr>
<th>Compound Name</th>
<th>Retention Time (min)</th>
<th>Molecular Mass (da)</th>
<th>Parent ions [M+H]^+</th>
<th>Parent ions [M-H]^−</th>
<th>Mass spectrum (m/z)^2</th>
<th>In-source fragments</th>
<th>Daughter ions^3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>dihydromyricetin^4</td>
<td>13.3</td>
<td>320</td>
<td>321</td>
<td>319</td>
<td>193, 125, 179, 301</td>
<td>192, 93, 181, 301, 151</td>
<td></td>
</tr>
<tr>
<td>sinapic acid</td>
<td>20.0</td>
<td>224</td>
<td>225</td>
<td>223</td>
<td>208, 164, 179</td>
<td>193, 208, 164, 149, 125</td>
<td></td>
</tr>
<tr>
<td>dihydroquercetin</td>
<td>22.6</td>
<td>304</td>
<td>305</td>
<td>303</td>
<td>285, 163, 125, 177, 275</td>
<td>125, 176, 285, 302</td>
<td></td>
</tr>
<tr>
<td>dihydrokaempferol</td>
<td>28.1</td>
<td>288</td>
<td>289</td>
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<td>93, 136, 151, 161, 257, 108</td>
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Y line extract (100% MeOH fraction)

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<th>Compound Name</th>
<th>Retention Time (min)</th>
<th>Molecular Mass (da)</th>
<th>Parent ions [M+H]^+</th>
<th>Parent ions [M-H]^−</th>
<th>Mass spectrum (m/z)^2</th>
<th>In-source fragments</th>
<th>Daughter ions^3</th>
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</thead>
<tbody>
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B line extract (total extract)

---

^1 retention time (min) according to PDA-detection on RP-HPLC, system ‘C’

^2 the most abundant fragments (>25% relative abundance), arranged in order of percent relative abundance; in-source fragmentation shown for [M-H]^− only

^3 fragmentation patterns relate to the parent molecular ion [M-H]^−; [M+H]^+ parent ions do not fragment well and daughter ions are not shown

^4 dihydromyricetin was present in trace amounts only and was detected by SIR

^5 ME, methyl ester, an artifact of the extraction (e.g. a hydrolysis product of sinapine)

^6 determined by SIR in the B line using the 50% acetonitrile gradient method
<table>
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<tr>
<th>Compound Name</th>
<th>Retention Time$^1$ (min)</th>
<th>Molecular Mass (da)</th>
<th>Parent ions</th>
<th>Mass spectrum (m/z)$^2$</th>
<th>In-source fragments</th>
<th>Daughter ions$^4$</th>
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---

$^1$ retention time (min) according to PDA-detection on RP-HPLC, system 'C'

$^2$ the most abundant fragments (>25% relative abundance), arranged in order of percent relative abundance; in-source fragmentation shown for [M-H]$^-^2$ only

$^3$ fragmentation patterns relate to the parent molecular ion [M-H]$^-^2$; [M+H]$^+$ daughter ions are not shown

$^4$ determined using the 50% acetonitrile gradient method
[M-H]$^-$ (parent) ion of 237 (an addition of 15 mass units [equal to -CH$_3$] to the [M-H]$^-$ of deprotonated sinapic acid) (3) in-source fragmentation producing a [M-H]$^-$ of 222 (equivalent to a deprotonated sinapic acid and representing the loss of the methoxy group) and daughter ions similar to the fragmentation pattern of sinapic acid (207, 208) (Table 4.6).

In contrast to seed coat extracts from the Y line, equivalent B line preparations contained fewer UV-visible compounds except for the sinapic acid and sinapic acid methyl ester (Figure 4.12, B). Seed coat preparations spiked with $\tau$-cinnamic acid and dihydroquercetin confirmed that dihydroflavonols and $\tau$-cinnamic acid did not appear to accumulate in the seed coats from the B line (data not shown). Total ion current scans of the B line extract for molecular ions of a specific mass, designed to detect $\tau$-cinnamic acid (148 da) or specific dihydroflavonols (dihydrokaempferol [288 da], dihydroquercetin [304 da], dihydromyricetin [320 da]) did not display any compound with comparable masses at the appropriate RT. A search of the TIC for characteristic in-source molecular ion fragments and for daughter ions was also made for each compound of interest over a narrow time-window that corresponded to the UV-detection-based retention time of the authentic standard. In addition, the B line extract was re-separated under SIR mode with the mass analyzer tuned to detect only the characteristic molecular ions of $\tau$-cinnamic acid and the dihydroflavonols. This mode significantly increased the sensitivity of the instrument for the specified compound. Under these conditions, there were no compounds in the B line extract that could be identified as $\tau$-cinnamic acid or as a dihydroflavonol.

Flavonols were identified by UV- and mass spectra in both Y line and B line extracts, although the B line contained much lower amounts. The relative abundance
of flavonols in *B. carinata* appeared to occur in a pattern similar to the dihydro-
flavonols, because quercetin was more abundant than kaempferol in the Y line
preparation and myricetin was undetectable in both UV- and mass spectral data (Figure
4.12 A). Trace quantities of quercetin and kaempferol were detectable only by SIR (as
301 and 285 m/z [M-H]⁻) in the B line extract; molecular ions for myricetin were not
found in the B line extract by SIR. Flavonols had not been detected in any of the hand-
dissected seed coat preparations. Sinapic acid (either as a free acid or methyl ester)
was present in the highest concentration of all the UV-detectable phenolics in both Y
and B lines as measured by PDA and MS-MS. The difference in RTs of sinapic
acid/methyl ester in the B line analysis compared to the Y line (Figure 4.12) related to
differences in sample matrix. One sample represented the un fractionated extract (B
line) and the other sample represented the 100% MeOH fraction (Y line). Authentic
standards were separated in each sample set (section 3.10.2) and run-to-run changes in
the RTs of these standards corresponded to the changes in the RT of compounds in the
seed coat extracts (data not shown).

Two broad UV-detectable peaks with co-eluting compounds (RT 3.9 and RT
11.0) were detected in the seed coat preparation of B line tissue (Figure 4.12, B). Base
hydrolysis of B line seed coat extract reduced the UV-detectable compound(s) at both
these retention times from 2.5 and 0.8 AU to less than 0.2 AU (data not shown).
However, a concurrent absorbance increase in another UV-detectable peak was not
observed. Basic-hydrolysis of the Y line seed coat extract (total fraction) did not
appear to increase the absorbance of any of the UV-detectable components. Sinapine
was not detected in the hand-dissected seed coat extracts, but it was detected in the
water and 30% MeOH fractions of the mechanically-collected seed coat tissue of the Y
line and the unfraccionated B line by UV-illumination of the extracts separated by 2D-TLC (data not shown).

Molecular ions of the phenylpropanoid standards (e.g. sinapic and \( \tau \)-cinnamic acids) were detected in both NI and PI modes but in-source collision cell fragmentation patterns were more evident in NI mode (Table 4.7). When the MS scans were in the PI mode, the intensity (% abundance) of the molecular ion \([M+H]^+\) was low and fragments less than 100 m/z were abundant for all standards tested. The signal intensity of the daughter ions of the flavonol standards (myricetin, quercetin and kaempferol) was augmented by increasing the gas pressure in the collision cell which had the effect of reducing the vacuum from \(3.6 \times 10^{-5}\) to \(8.1 \times 10^{-4}\) mBar and increasing the analyzer vacuum to \(2.1 \times 10^{-5}\) mBar. This increased argon concentration in the collision cell raises the chance of the selected ion colliding with the argon molecules and fragmenting to generate daughter ions for detection in the second analyzer.

Although both NI ([M-H]⁻) and PI ([M+H]⁺) mode generated mass spectra of compounds present in the Y and B line extracts, detection intensities were stronger using the NI mode to determine flavonoid molecular (parent) ions and fragmentation patterns in seed coat extracts. The conditions developed for the flavonol standards were applied to the seed coat samples to detect the flavonols. The flavonols appeared to be very stable and did not readily fragment. The dihydroflavonols were of a more fragile nature and fragmented very readily. Overall, the NI mode was more useful for determining both molecular and daughter ion fragmentation patterns. The most common fragments were due to loss of a water molecule (18 m/z), methyl group (15 m/z) and/or carbon dioxide (44 m/z).
4.7 RFLP analysis of genomic DNA from *B. carinata* siblings

Several RFLPs were detected on Southern blots when the DNA of 'S<sub>1</sub> siblings' and Y and B lines was hybridized with a genomic DNA sequence from *CHS* (Figure 4.13). One polymorphism generated a band of ca. 3.0 kilobases (Kb), that was evident in the DNA from the B line and 16 out of 22 samples of the S<sub>1</sub> brown-seeded siblings. The DNA from the Y line displayed no fragment of this size, and out of 13 yellow-seeded S<sub>1</sub> siblings, ten were also missing the 3.0 Kb fragment (indicated by '()', Figure 4.13, A and B gels).

Additional *CHS*-related polymorphic bands, ranging in size from 5.0 to 9.4 Kb, were present between the Y and B lines and the S<sub>1</sub> siblings. The B line DNA and DNA of 11 brown-seeded siblings contained at least three fragments each that were missing entirely from both the Y line and the yellow-seeded S<sub>1</sub> sibling DNA (indicated by '()', Figure 4.13, gel A). Although DNA on the second Southern blot with 18 sibling lines blotted less clearly in this region, it was possible to observe that there were similar RFLP differences in the DNA of Y and B lines as well as sibling DNA for polymorphisms in this size range (Figure 4.13, gel B). RFLPs that were associated with the seed colour trait were not detected in the DNA of Y and B lines or the S<sub>1</sub> sibling DNA when hybridized with sequences derived from *PAL* and *CAD* or from digests with *BamH*I, regardless of the probe. Digests with *Hind* III produced very small fragments and no RFLP was associated with the seed colour trait.

4.8 Detection of selected flavonoid gene transcripts in *B. carinata* Y and B lines

4.8.1 Northern analysis of RNA extracts from developing seed

Two RNA extraction methods were used in this research, 'hot phenol' and a commercial kit (RNeasy, Qiagen). Although the hot phenol method worked very well
Figure 4.13 RFLP analysis of DNA from Brassica carinata PGRC/E 21164, digested with EcoRI and probed with a CHS sequence.

'⇒' indicates RFLP between Y and B DNA and S₁ siblings in the 3 Kb region; '⇒' indicates fragments in the 5.0 to 9.4 Kb region.

(A.) Y line (lane 1) and six yellow-seeded S₁ siblings (lanes 2-7); B line (lane 9) and 11 brown-seeded S₁ siblings (lanes 10-20).

(B.) Yellow-seeded S₁ siblings (lanes 1-7); brown-seeded S₁ siblings (lanes 9-19); (lane 8, empty on both gels A and B).
for isolating satisfactory amounts of total RNA (ca. 50 to 80 mg g\(^{-1}\) seed tissue), a viscous substance thought to be a polysaccharide (Wilen et al., 1990), co-purified with the 20 and 30 dap stages of seed development and was difficult to remove. Precise volumes of RNA were difficult to measure in viscous samples unless the RNA preparation was diluted 2-fold, centrifuged for 5 min at >10,000 rpm and then immediately pipetted to a new tube. Satisfactory yields of RNA from commercial RNA purification kits were achieved with developing seed from early stages (0, 5, 10 dap), seedling tissue and apical meristem tissue. Unknown factors in later seed stages (20, 30 dap) interfered with the isolation of RNA using these kits, so the hot phenol isolations continued to be used for these later seed stages.

*PAL*, *CHS* and *CAD* were only detectable by probing Northern blots of total RNA from seedling leaf tissue (positive control) but not with developing seed (0, 5, 10, 20 and 30 dap) (data not shown). In a subsequent Northern blot with RNA of developing seed, there also was no detectable signal using a *DFR* probe. A detectable signal was observed when the Northern blots were probed with actin (data not shown).

### 4.8.2 Expression of *DFR* in developing seed of *B. carinata*

The presence of dihydroflavonols in seed coat of the Y line indicated that the substrate for DFR was accumulating. Consequently, RT-PCR assays were used to determine *DFR* gene expression patterns in developing seed because these reactions have a more sensitive detection limit for mRNA than Northern hybridizations. The PCR amplifications produced only one fragment of ca. 200 bp in each PCR reaction using primers based on the *DFR* sequence of *A. thaliana* and *B. carinata* (Appendix G4). PCR products appeared to be reduced in all stages of the developing seed of the Y line compared to the same stages in developing seed of the B line (Figure 4.14, A).
Figure 4.14. Gel A. Expression of DFR in developing seeds of *Brassica carinata*.

Lanes marked '0' to '30', dap, developing seed. Lanes 'L1' and 'L2' warm-grown seedling leaf tissue, from B and Y lines (respectively); Lane marked*, empty.

Figure 4.14 B. Southern blot of Gel A hybridized with DFR.

Samples correspond to dap, as in Gel A. Lane* empty.

Figure 4.14 C. RT-PCR with actin primers.

Actin primers produced 650 bp and 150 bp fragments. cDNA template from same RT reaction as RT-PCR for DFR reactions. Arrow (⇒) indicates ≤100 bp fragment from 5' primer only (see lane 6 in Fig. 4.14, D). Size ladder (bp) is ‘1Kb-Plus’ (Gibco-BRL).

Figure 4.14 D. Control RT-PCR with DFR and/or actin primers. 

Lane 2, both DFR and actin primer sets present, no cDNA; lane 3, 3' primers only, no cDNA; lane 4, 5' primers only, no cDNA; lane 5, cDNA and both DFR and actin 3' primers; lane 6, cDNA and both DFR and actin 5' primers. Arrow indicates 100 bp fragment amplified from 5' primer. Size ladder (bp) (lane 1), ‘1Kb-Plus’.
Hybridization of a radioactive DFR probe (Appendix F.2) to the 200 bp band in a Southern blot of the RT-PCR-amplified DNA confirmed that the sequence amplified during the PCR reaction was homologous to DFR. The radioactive signal accentuated the visualization of the overall lower level of transcript in the Y line compared to the levels in the B line in which the quantity of DFR transcript had evidently reached the maximum level of accumulation by 10 dap in the developing seed (Figure 4.14, B). This amount appeared to be maintained in the B line until at least 20 dap, decreasing by 30 dap (Figure 4.14, A and B).

Independent RT-PCR with actin primers (conducted in the same thermal cycler with the DFR assays, with cDNA template from the same RT reaction), produced fragments at 650 bp (Figure 4.14, C). These fragments appeared to be amplified equally in all the samples and verified that equal amounts of RNA were used in all assays. When primers for both actin and DFR were combined in a duplex PCR reaction, amplification conditions could not be established where both primers performed optimally. Disparate primer melting temperatures (67-68°C, actin and 51-55°C, DFR) were thought to contribute to this technical problem. Control assays with the cDNA and one primer alone (either 3' or 5', DFR and actin) and a reaction with both sets of primers but without a cDNA template produced no detectable bands at either 200 or 650 bp (Figure 4.14 D). In the actin PCR reactions, two other fragments were produced (150 bp and at <100 bp, Figure 4.14, C). Faintly-amplified fragments appeared in some of the control reactions at ≤100 bp when the 5' actin primer was present (Figure 4.14, D, lanes 2 and 6). It appears that the 5' actin primer may be able to anneal to other sequences under the PCR conditions used here and inefficiently
amplify small fragments, in addition to the expected fragment at 650 bp. Since the cDNAs from the first strand synthesis were very prone to breakage, cDNA samples were discarded after 2 weeks. In addition, there was non-specific priming and amplification that produced small (≤100 bp) fragments (Figure 4.14 D).

4.8.3 Expression of DFR in seedling leaf tissue of B. carinata

Since anthocyanin biosynthesis appeared to be different in the Y line seedlings compared to B line seedlings grown in warm temperatures (section 4.4.2), DFR expression was examined from warm- and cool-grown seedlings. Initially, RT-PCR assays with cDNAs produced from total RNA of warm-grown seedlings were observed to have less DFR transcript (represented by a fragment at 200 bp) compared to the amount seen in the B line preparations (Figure 4.14, A, lanes L₁ and L₂, respectively). On a Southern blot, the cloned DFR sequence hybridized to the 200 bp fragment thus indicating that it contained a DFR-related sequence amplified by PCR (Figure 4.14, B, lanes L₁ and L₂, respectively). The cloned inserts of the 200 bp fragments from several PCR reactions submitted for sequencing were homologous to published sequences of DFR found in A. thaliana (data not shown). Actin was used as a quantitative control assay, described previously for the experiments with developing seed (section 4.8.2).

Subsequently, Northern blots of total RNA leaf extracts of two sets of Y and B line seedlings were performed (cotyledon stage to the third-leaf-emerged stage, two temperature regimes, Appendix D.4). Expression of DFR was analyzed by densitometry and normalized by the value of the respective actin sample that corresponded with the same leaf stage and temperature regime (Table 4.8; Figure 4.15). In cool-grown seedlings, the level of DFR transcripts of the Y line was very similar at
all seedling stages (Table 4.8; Figure 4.15). While the expression level appears to be relatively constant in the cool-grown Y line seedling leaves and cotyledons, DFR transcripts in the B line appear to increase from the cotyledon stage to the third leaf stage, ultimately producing more than twice as much DFR transcript than the Y line in the third leaf stage. The relatively constant high level of expression of DFR in the Y line leaves and the rapid increase of transcript in the B line is consistent with the uniform appearance of the leaf colour observed in cool-grown seedlings at the second to third leaf stage.

In the seedlings grown at warm temperatures, the transcription of the DFR gene is much lower in the Y line compared to the level of expression in the B line (Figure 4.15) and the normalized expression level of DFR in the seedlings was shown to be highest at the cotyledon stage (Table 4.8). This was consistent with the observation that pigment was visible in both lines in the cotyledons grown under warm conditions (section 4.41). Increased DFR expression in the B line and reduced expression in the Y line for all three leaf stages from warm-grown plants corresponded with the presence/absence of anthocyanins reported earlier from iso-amyl alcohol extracts (Figure 4.5).
Table 4.8  Temperature effects on the expression of *DFR* in seedlings of *Brassica carinata* PGRC/E 21164, Y and B lines.

<table>
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<th>Sample</th>
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<th>Actin signal Sample</th>
<th>Density</th>
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<sup>1</sup> optical density (OD) mm<sup>-2</sup>

<sup>2</sup> B, B line; W, warm growth environment (20 – 25°C); Ct, cotyledon stage

Y, Y line; C, cool growth environment (15 – 18°C)
A. Northern blot probed with DFR

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- B line tissue
- Y line tissue

- Warm-grown seedlings
- Cool-grown seedlings

B. Northern blot probed with actin

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- B line tissue
- Y line tissue

- Warm-grown seedlings
- Cool-grown seedlings

Figure 4.15 Northern blot of total RNA extracted from *Brassica carinata* seedling leaf tissue grown in warm (20 to 25°C) and cool (15 to 18°C) environments.

A. Cotyledon (Ct) and leaf tissue (first leaf fully emerged, 1, to third leaf fully emerged, 3) probed with DFR. Blot exposed to film for 48 h.

B. Blot A probed with actin after stripping DFR probe; blot exposed to film for 40 h.
CHAPTER 5

DISCUSSION

The genetics and enzymology of phenylpropanoid and flavonoid biosynthesis have been studied intensively for many years and more than 3,500 flavonoids have been described (Harborne, 1975). Knowledge of a highly regulated, biochemically complex pathway has emerged, assisted by research using numerous mutants in petunia, snapdragon, maize, Arabidopsis and other genera (reviewed in Hahlbrock and Scheel, 1989; Holton and Cornish, 1995; Mol et al., 1996).

In the research reported here, new information about the occurrence of phenylpropanoids and flavonoids in the Brassicaceae is presented along with documentation of DFR expression, an important target for regulation by transcription factors. A comparison of the phenylpropanoid and flavonoid profiles of the seed coat and of DFR expression in developing seed and seedling leaves was possible using near-isogenic lines of yellow- and brown-seeded B. carinata PGRC/E 21164. The results of this biochemical and genetic investigation demonstrate that key changes in flavonoid metabolism occurred to produce the yellow-seeded phenotype. Such changes are likely the result of down-regulation of the DFR gene. The conclusions arising from this research have broad implications for investigations using Brassica spp. germplasm to develop canola with a low fibre, unmottled, yellow-seeded phenotype.

5.1 Two different pigments contribute to the dark seed coat of the Brassicaceae

Seed coat pigmentation in the Brassicaceae appears to be under complex genetic
control because there are several variations in both the location (hilum, random patches, entire) and the type of pigment present (condensed tannin, phlobaphene).

Histochemical analysis of pigments in the seed coat of mottled- or dark-seeded Brassicaceae revealed that the majority of the unextractable seed coat pigment was due to condensed tannins rather than the anthocyanins, which are commonly reported to colour the seed coat in legumes. The kinetics of red stain formation in the seed coat of brown- and black-seeded samples of the Brassicaceae, in isolated patches and around the hilum of the yellow-seeded samples distinguished between condensed tannin pigmentation (slow anthocyanidin formation) and anthocyanins (rapid formation), similar to discrimination between these pigments in legumes (Lees et al., 1993; Skadhauge et al., 1997).

In situ histological staining was the only way to identify condensed tannins in seed coat tissue of the Brassicaceae because of the difficulty in extracting the pigment from ripened seed. Most of the pigment was unextractable by the methods used here to remove the visible pigments from mature seeds. This observation, consistent with other studies that also reported unextractable pigments in the Brassica seed coat. Whole seeds of B. rapa cv Echo (purple-black seed colour) treated with acidified MeOH did not release any pigment and the coloured material remained bound to the seed coat fraction (Durkee, 1971). When these B. rapa samples were soxhlet-extracted, the extracts contained only trace amounts of leucoanthocyanidin (an uncoloured condensed tannin precursor) and the pigment remained bound in the seed coat tissue. The red pigment in the seed coat of dark red kidney bean (P. vulgaris cv Montcalm, carrying the recessive allele ‘rk’) was unextractable and remained in the original material during the flavonoid isolation procedure (Beninger and Hosfield, 1999).
Subsequent analysis of this kidney bean seed coat (using the vanillin assay) confirmed that this red pigment was a condensed tannin. Beninger and Hosfield (1999) speculated that the normally colourless condensed tannins undergo secondary changes during seed maturation to form complexes with other phenolics in the seed coat; these complexes subsequently darken. This darkening process was also proposed for condensed tannins in the brown testa of the caryopses of sorghum (Stafford, 1990).

Histochemical treatment showed that in addition to condensed tannin, a phlobaphene-type of pigment was present in several of the brown-seeded Brassicaceae examined in this study (Table 4.1, B. carinata, cv S-67 and brown-seeded S2 line; B. napus KF95-progeny; Sinapis alba SRS 2495; S. arvensis). This pigment remained as unchanged brown patches after the rest of the seed coat had stained red for condensed tannins in the acidified organic solvents. It is probable that the compound causing brown colouration was chemically different from condensed tannin because the brown areas did not change colour, even after 96 h of treatment with acidified histochemical. This pigment contributed to the spotty or mottled colour of the seed coat in these accessions. Inert, unextractable brown to brown-red pigments deposited in a patchy or entire pattern in the seed coat of soybean, maize and common bean (P. vulgaris) were suggested to be phlobaphenes (Bernard and Weiss, 1973; Styles and Ceska, 1989; Bassett, 1995). A phlobaphene-like pigment was also present in small spots or large patches in two lines of B. napus (progeny KF95-1A5 and -4E4), possibly in B. napus cv Apollo (which was otherwise stained very dark red) and in the seed coats of some yellow-seeded material that did not stain red (B. carinata, S2 generation and Y line; B. juncea cv AC Vulcan and cv Lethbridge 22A). It is possible that the light brown seed coat and the hilum of Eruca sativa, as well as the dark brown seed coat and hilum of
Crambe abyssinica cv Prophet, also contain the same phlobaphene-like pigment throughout their seed coat. The seed coat tissue in these species did not produce a red stain, indicating that condensed tannins were absent.

Unextractable pigments from the black seed coat of B. napus cv Tower (Leung et al., 1979) and from the beige, brown and red seed coats of three broad bean cultivars were reported to be difficult to characterize (Nozzolillo and Ricciardi, 1992). Of the extractable component, only simple phenolic acids (sinapic, ferulic, caffeic and coumaric) and traces of leucocyanidin were identified in B. napus cv Tower, and flavan-3-ol units were obtained from an unidentified parent compound in broad bean (Leung et al., 1979; Nozzolillo and Ricciardi, 1992). These authors could not determine the precise chemical nature of the unextracted pigment in either case, but histochemical staining of the dissected seed coat with vanillin-HCl or BuOH-HCl would have provided useful information.

Although anthocyanin deposition was not detected in either B. carinata seed coat material or in seed coats of the other Brassicaceae studied, there might be anthocyanins present at such low levels that the pigment was undetectable by the methods used here. Anthocyanins can certainly be produced in other tissues because red-tinged siliques, leaves and red-purple stems are often seen in field-grown material and anthocyanins have been isolated from the vegetative tissue of Raphanus spp., B. oleracea and B. napus (Hodges and Nozzolillo, 1996). However, data from the in situ staining experiment described here constituted evidence that these histochemicals can successfully be used to discriminate between anthocyanins and condensed tannins for a broad range of Brassicaceae. Histochemical examination of seed coat tissue confirmed
that the dark-seeded and mottled samples of the Brassicaceae contained only two end-products of the flavonoid pathway, condensed tannins and phlobaphenes.

5.2 Transcriptional down-regulation of DFR reduces seed coat pigment biosynthesis in *Brassica carinata*

In the preceding section, seed coat pigmentation in the Brassicaceae was suggested to be under complex genetic regulation because there were two identifiable pigment compounds and variable patterns of pigment distribution. The dominant one-gene model of inheritance of the 'yellow seed' phenotype in *B. carinata* was attributed to a repressor mechanism that inhibited the expression of seed pigmentation (Getinet and Rakow, 1997). Although a structural gene for down-regulation was not identified, Getinet and Rakow (1997) explained that two or more loci could be involved in pigment biosynthesis because of the amphidiploid nature of *B. carinata* and that the number of loci affected by the repressor would not be apparent from the segregation ratios they reported for a one-gene model. These suggestions are consistent with the observations reported in the present research.

These discoveries are indicative of control by unknown regulatory factors such as described in maize and legumes (Dooner, 1983; Goff *et al.*, 1992; Tuerck and Fromm, 1994; Majnik *et al.*, 1998). In legumes, regulatory proteins homologous to those found in maize control the presence of a pigmented saddle region and a coloured hilum or a complete absence of pigment in seed coats (Bernard and Weiss, 1973; Bassett, 1995; Beninger *et al.*, 2000). The pigment controlled by the maize and legume regulatory loci was generally identified as an anthocyanin. However, in one case the recessive 'rk' gene activated condensed tannin formation in the seed coat of a dark red kidney bean and anthocyanins were not formed (Beninger and Hosfield, 1999). In
contrast, presence of the banyuls (ban) mutation in Arabidopsis was associated with the precocious accumulation of high levels of anthocyanins within the inner layer of the seed coat compared to the WT, which normally accumulated condensed tannin (Albert et al., 1997; Devic et al., 1999). The lack of anthocyanins in WT Arabidopsis (when BAN is active) may be evidence of repression of a regulatory factor affecting the BAN locus (analogous to LAR, and specifically involved in condensed tannin formation) early in seed coat development because the WT BAN allele was expressed in seed coats at a very early stage of integument development, but not prior to fertilization (Devic et al., 1999). No transcripts from the (mutated) ban allele were detectable in the developing seed after the globular stage. Nesi et al. (2000) have cloned and analyzed the tt8-3 locus which encodes a bHLH-domain protein. Their experiments demonstrated that TT8 is required for normal expression of DFR and BAN.

In a different study of Arabidopsis, the ast mutation (from 'Arabidopsis spotted testa') has been described as a single recessive mutation responsible for spotty anthocyanin accumulation in the immature seed coat (Tanaka et al., 1997). It was not clear from the published report whether the chemical basis used for pigmentation identification was accurate. However, ast may be allelic to the ban locus. Comparable classes of regulatory genes and multiple alleles at loci similar to those described for maize and legumes (e.g. the rk and I loci, section 2.4), as well as the BAN and ast alleles in Arabidopsis, could be affecting the pattern of condensed tannin and phlobaphene deposition in the seed coat of many species of the Brassicaceae, especially those species of an amphidiploid nature.

The accumulation of dihydroflavonols, the substrate for DFR, together with differences in the abundance of DFR transcripts in the developing seed of B. carinata
Y and B lines, suggest that the structural gene DFR is at least one of the loci targeted in the flavonoid pathway for down-regulation. Transcript levels of DFR were much lower in developing seed of the yellow-seeded line and did not reach levels comparable to those in developing seed of the B line. These differences in transcript abundance suggest that changes in regulatory factors in the Y line result in reduced DFR expression. This could be due to the reduced affinity of a regulatory protein for the DFR promoter resulting in decreased pigment biosynthesis in yellow-seeded B. carinata.

Further to the ‘one-gene’ model of seed colour inheritance, mutations in a gene that encodes a regulatory factor influencing DFR expression, or alterations in untranslated regions of DNA of the DFR gene, are candidates which would fit the circumstances of pigment regulation suggested by Getinet and Rakow (1997). In addition, B. carinata F1 seed was noted to be a darker shade of yellow (section 2.4.3), leading these authors to suggest that the gene was incompletely dominant. These suggested mutations are consistent with incomplete dominance if, for example, the promoter upstream from DFR was altered, decreasing the affinity of a DNA-binding protein. In this example, the least pigmented seed would thus be produced only by plants homozygous for the allele altered in the DFR promoter region. More pigmentation would occur in seeds from heterozygote plants, since heterozygotes would carry at least one allele with slightly better binding affinity. Separating the homozygotes from the heterozygotes would be challenging because the progeny seed must be evaluated for subtle shades of ‘yellowness’. In a situation such as this, a codominant molecular marker for the mutation would be invaluable.
The gene 'AtMYC1' is one possible candidate for the suggested regulatory protein that could control pigment biosynthesis in *B. carinata*. This *A. thaliana* gene encodes a MYC-related protein that functions as a seed-specific transcription factor involved in pigment production (Urao et al. 1996). *AtMYC1* and its corresponding cDNA encode a MYC-related protein that has a bHLH motif at the C-terminus highly homologous to the B/R transcription factor family, later shown to activate phlobaphene and anthocyanin synthesis in maize (Ludwig et al., 1989; Sainz et al., 1997). The role of *AtMYC1* fits the model for seed coat pigment regulation in *B. carinata*.

### 5.2.1 Pleiotropic effects of down-regulated DFR expression

In this research using near-isogenic Y and B lines of *B. carinata*, there was indirect evidence that anthocyanin biosynthesis in leaf tissue of warm-grown Y line seedlings was down-regulated. Under warm conditions, anthocyanin production ceased in leaves of the Y line soon after the first leaf emerged and these seedlings lost the blue-green appearance that was visible continuously in the B line. Very little anthocyanin could be extracted into iso-amyl alcohol from the Y line seedlings that had been grown in a warm environment, in contrast to extraction of the B line tissue (section 4.4). In cooler growth conditions however, there was no apparent difference in leaf pigmentation between the Y and B line. Temperature effects on anthocyanin content have been observed in other species of *Brassica*. In leaves of oilseed rape (*B. napus* var. *oleifera* L.) grown at 2°C for 3 weeks, a two-fold accumulation of anthocyanins was observed, compared to plants grown at warmer temperatures without a period of chilling (Solecka et al., 1999), unlike the B line of *B. carinata*.

Concurrent with the decrease in anthocyanin content, the expression of *DFR* in the Y line was reduced compared to the B line, when both were grown at 20 to 25°C.
Down-regulation of DFR under these conditions suggests several possibilities for transcription factor interactions. For instance, the binding affinity of one of the family of DFR transcription factors (e.g. MYC/MYB proteins) for anthocyanin synthesis could have a temperature sensitive affinity to the promoter region from DFR in the Y line. In cooler growing conditions, DFR transcripts are produced and the anthocyanin biosynthetic pathway is not impeded. Alternatively, a different chilling-induced transcription factor could be involved that enhances DFR expression by a positive interaction with DFR-related regulatory controls so that, again, pigment is produced. If a temperature-sensitive regulator is involved and the absence of a regulatory protein disrupts anthocyanin production in the seedling leaves of yellow-seeded B. carinata at warmer temperatures, developmental stage must play a part in the responsiveness of DFR, since this was an age-dependent process. Clearly, as the seedlings grew past the five-leaf stage, there was enough DFR expression to permit pigment biosynthesis to proceed. Other researchers have reported temperature-related effects on pigment biosynthesis in B. napus. The colour of the seed produced by DH populations of B. napus changed from yellow to dark brown when the temperature regime at which the plants were grown was reduced from 24/20°C to 16/12°C (Van Deynze et al., 1993). Black-seeded populations were not affected by the cooler growing conditions. The darkened yellow colour of seed ripened on cool-grown B. carinata was similarly noted for its changed appearance compared to seed harvested from warm-grown plants (section 4.1).

Many Brassicaceae, including B. rapa, A. thaliana, B. napus, B. oleracea and Raphanus spp., transiently display red to purple pigmentation in the cotyledons and stems immediately following germination (Stringam, 1971; Kubasek et al., 1992;
Hodges and Nozzolillo, 1996). Normally, as the seedlings grow to the fifth-leaf stage and older, this early appearance of pigmentation disappears from the leaves. In *B. carinata*, there was also evidence of age-related anthocyanin biosynthesis. At the cotyledon stage, stems and cotyledons were tinged with purple-red. *DFR* transcripts were more abundant at this stage than at any other time, except in the case of the cool-grown B line, where the *DFR* transcripts increased consistently over the sampling time (Table 4.8). A similar age-dependent induction of transcripts related to anthocyanin biosynthesis has been reported for *Arabidopsis* (Kubasek et al. 1998), maize (Dooner, 1983) and rice (*Oryza sativa* L.) (Reddy et al., 1994) as well, with peak abundance occurring between 1 and 4 d after germination. These observations suggest the possibility that more than one protein may be involved in controlling tissue pigmentation in *B. carinata* and that these are differently affected. Perhaps environmental factors control the expression of one factor and the developmental stage of the plant affects the expression of another regulator.

The physiological basis for transient activation of flavonoid metabolism such as occurs in a defined period of early plant development is unknown. Differences in *DFR* transcription between the Y and B lines in developing seeds and leaves may be a pleiotropic effect related to developmental events of the metabolic pathway for pigment production. Pleiotropic effects on seed coat traits have been reported for other species of *Brassica* including *B. juncea*, *B. rapa* and *B. napus* (Woods, 1980; Schwetka, 1982; Shirzadegan and Röbbelen, 1985). Traits such as lower fibre, higher oil concentration and larger seed size were associated with the unpigmented seed coat trait in these species. The reduced metabolic demand for phenylpropanoid and flavonoid substrates by virtue of the unpigmented seed trait could explain why such traits were pleiotropic.
Fibre includes lignin, a phenylpropanoid-derived polymer, and fatty acid synthesis for oils requires malonyl-CoA that might be used otherwise in flavonoid metabolism by CHS. The reduced anthocyanin in the leaves of yellow-seeded *B. carinata* could be a pleiotropic effect that is associated with a temperature-sensitive regulator of the flavonoid pathway. Lack of anthocyanin pigmentation in young seedlings grown under specific conditions might serve as a selectable marker for the yellow-seeded trait.

### 5.2.2 Development of unmottled yellow-seeded phenotypes in *Brassica*

The inheritance of seed coat pigmentation in *Brassica* species has been the subject of many investigations (section 2.4.3). Generally, these studies have focused on seed coat colour in its entirety rather than separating the genetics of hilum colour and patterned pigmentation or considering that more than one class of pigment could be produced, thereby involving more than one enzymatic pathway. Interpretation of data from *B. napus* crosses, for instance, might be improved by including the type of pigment (condensed tannin, phlobaphene) and pattern of seed coat pigmentation (*e.g.* pigmented hilum, spotty or mottled colouring) in the analysis. The undesirable variation in seed colour (dark brown, red-brown, yellow-brown) and the mottled pigment deposition that was reported in the progeny of *B. napus* YN90-1016 x cv Apollo (KF95 series) is an example of the complexity of the seed coat pigmentation genetics involved. The pigmented patches in two of the progeny were identified as having only condensed tannin and in another two as having only phlobaphene-like pigment (Table 4.1). One seed sample (KF95-5C8) appeared to be variably mottled with both kinds of pigment. By observing segregation ratios of pigment type in the F$_2$ (seed coat) of *B. napus* YN90-1016 x cv Apollo, the number of loci and their relationship to one another could be determined.
Genetic studies of pericarp and seed coat pigmentation of maize, common bean and soybean have been facilitated by the development of breeding lines representing each of the multiple alleles at different loci. Allelic interactions and their effect on the type of pigment and deposition pattern in these lines were important to the overall understanding of the genetic control of seed coat (in legumes) and of pericarp (maize) colour. Comparable breeding lines in \textit{Brassica} species could assist in the development of unmottled yellow-seeded phenotypes in \textit{Brassica} species that would be useful in the improvement of canola.

Criteria for such \textit{Brassica} breeding lines should include the properties of deposition pattern and chemical character of the seed coat pigment. These breeding stocks would each then be selected for a specific variant of the gene(s) controlling the pattern of pigmentation or pigment type in the seed coat. To this end, characterizing and sequencing the different variants is essential. Germplasm from different origins may be a source of different alleles for the study of the control of seed coat pigments and patterns (\textit{e.g.} pigmented hilum, patchy or mottled colouring). It is possible that seed stocks from Europe may have factors controlling seed coat pigmentation dissimilar to those in the breeding stocks from India or China and, as such, a geographically wide range of material should be investigated.

5.3 \textbf{Disruption of dihydroflavonol reductase (DFR) enhances the accumulation of metabolic intermediates}

It was suggested in earlier sections that seed colour inheritance in \textit{B. carinata} was under complex genetic control. Gene expression that enabled flavonoid end-product synthesis (condensed tannin, phlobaphene) was suggested to be regulated by transcription factors. In one model, down-regulation of \textit{DFR} would enhance the
accumulation of pre-DFR metabolic intermediates because flavonoid biosynthesis was
terminated or severely reduced before the branch point to condensed tannins and
phlobaphenes in yellow-seeded *B. carinata*. Consistent with this model, specific,
colorless phenylpropanoid and flavonoid products (dihydroquercetin, quercetin and *t*-
cinnamic acid) from earlier enzymatic steps were relatively abundant in extracts of seed
coats of the Y line. Other intermediates (dihydromyricetin and kaempferol) were less
abundant. By comparison, neither *t*-cinnamic acid nor any of the dihydroflavonols
appeared to accumulate in brown-seeded *B. carinata*. Trace amounts only of quercetin
and kaempferol were present in the B line.

Phytochemical analyses of other genera with seed colour mutations have also
shown an accumulation of metabolic intermediates. Dihydroquercetin (a
dihydroflavonol), kaempferol and quercetin (flavonols) accumulated in light-seeded *tt3*
and *tt8* mutants of *A. thaliana* in both seedling tissue and seeds (Shirley *et al.*, 1995;
Pelletier *et al.*, 1999). Flavones and flavonols can accumulate in the seed coat in the
common bean (*P. vulgaris*) as stored products and Beninger and Hosfield (1998, 2000)
proposed that these were the agents responsible for the pronounced yellow seed colour
in some bean cultivars. In a study with two *Vicia faba* cultivars, it was found that the
seed coats of the dark-seeded cultivar, Alfred, contained only condensed tannin and
that seed coats of the white-seeded, tannin-free cultivar, Blandine, contained flavones,
flavonols and dihydroflavonols (Bekkara *et al.*, 1998).

Since it is apparent that extracts from yellow-seeded *B. carinata* contain more
dihydroflavonols and flavonols than those from brown-seeded material, these
compounds may contribute to the yellow appearance of the *B. carinata* seed in the
same way that accumulation of flavonols and flavones was suggested by Beninger and
Hosfield (1998, 2000) and Bekkara et al. (1998) to provide the yellow colour of seeds in some legume cultivars. Preliminary analyses (TLC) of an accession of yellow-seeded *B. juncea* (developed from crosses to two Russian cultivars, Donskaja and Jubilejnaja) showed that flavonols and a dihydroflavonol were abundant in these seed coat extracts, suggesting that flavonols could be responsible for the 'yellowness' in seed of other *Brassica* species (e.g. *B. carinata* and *B. juncea* cv Lethbridge 22A, *B. rapa* cv AC Parkland).

Results from analyses of yellow- and brown-seeded *B. carinata* implied that both the phenylpropanoid and the flavonoid biosynthetic pathways were affected in the Y line. In addition, the lower concentration of lignin in yellow-seeded material indicated that there were changes in the biosynthetic pathway that used phenylpropanoids substrates to form this complex phenolic polymer.

**5.3.1 Flavonol accumulation is a consequence of disrupted end-product biosynthesis in the flavonoid pathway**

In the results reported earlier, quercetin and kaempferol (flavonols) were present in different amounts in the Y line seed coat extracts, with quercetin by far the most abundant. Myricetin was not detected in either Y or B line extracts. This was not unexpected because flavonols are produced from their respective dihydroflavonols by flavonol synthase (FS) (Gerats et al., 1982; Forkmann and Ruhnau, 1987). Analysis by LC-MS of Y line seed coat extracts demonstrated that dihydroquercetin was the most prevalent of the dihydroflavonols and that dihydromyricetin was present in trace quantities, detectable only by SIR. Thus, there was a very small amount of precursor in the substrate pool for FS to convert to myricetin. Shirley et al. (1995) used *A. thaliana* tt mutants to show that flavonols accumulated differently in the mutants (Table 2.1).
Quercetin was absent in some yellow-seeded *Arabidopsis* mutants and myricetin was absent in all the plants including the WT. Kaempferol was present in all the mutants except *tt4*, a CHS mutant, and variably detected in *tt5*, a CHI mutant.

The accumulation of flavonols in the Y line of *B. carinata* was probably influenced by the reduced enzyme activity of DFR. Flavonol synthesis would be enhanced as the metabolic fate of the dihydroflavonols, since this substrate could not be used for condensed tannin or phlobaphene biosynthesis in the Y line tissues. Flavonol synthesis as a metabolic alternative to pigment biosynthesis has been demonstrated in petunia mutants (Gerats *et al.*, 1982). There is considerable evidence that flavonols readily form in the place of anthocyanins in petunia flowers and that DFR competes with FS for a common pool of substrates (Gerats *et al.*, 1982; Forkmann and Ruhnau, 1987; Beld *et al.*, 1989; Quattrocchio *et al.*, 1993).

The data reported for mutant lines of petunia demonstrate that it could be very complicated to determine exactly why flavonols rather than anthocyanins form in some petunia flowers (section 2.2.1). In these mutants, DFR was active but presumably could not out-compete other enzymes for the dihydroflavonol substrate unless a kinetic advantage was genetically conferred by some means such as a regulatory factor. In *B. carinata*, enzyme activities for DFR, FS, F3’H and F3’5’H have not been measured to establish their kinetic relationship. However, it was evident in the Y line of *B. carinata* that flavonol synthesis was somehow affected because a large pool of dihydroquercetin accumulated even though quercetin was also detected. Since there were smaller amounts of dihydrokaempferol and kaempferol and no myricetin, it appears that yellow-seeded *B. carinata* may exhibit F3’H but no F3’5’H activity, similar to one of the petunia mutants described by Gerats *et al.* (1982) and Beld *et al.* (1989).
Theoretically, flavonol synthase in *B. carinata* would have unrestricted use of the dihydroflavonol substrate pool to synthesize flavonols if *DFR* expression was down-regulated and FS was functional. However, dihydroflavonols did accumulate so FS may be regulated in some manner as well. Some form of down-regulation could also be affecting the *F3’5’H* gene in the *Y* line as well. Alternatively, the *F3’5’H* gene may be mutated such that altered transcripts produced an enzyme with reduced activity. In either case, this would yield only trace amounts of dihydromyricetin, as was detected and explain why myricetin did not form in yellow-seeded *B. carinata*.

### 5.4 Polymorphisms in genomic DNA related to *CHS* and seed colour

Research with the *tt* mutants of *Arabidopsis* and with soybean having pigmented and unpigmented seed has shown that *CHS* can be implicated in the lack of seed coat pigmentation (Shirley *et al.*, 1995; Wang *et al.*, 1994; Todd and Vodkin, 1996). With this prior indication of the involvement of a specific locus in seed coat pigmentation, a *CHS* sequence was used as a probe on Southern blots of DNA from the *Y* and *B* lines and the *S1* generation to assay for genetic differences. A number of RFLPs were apparent in the offspring of the brown-seeded *S1* population and the *B* line. However, these polymorphisms were dominant for the pigmented seed trait and as such, it was not possible to distinguish between homozygous brown-seeded and heterozygous yellow-seeded individuals, both of which would display the allele with the restriction site. Such heterozygosity would account for the presence of restriction sites associated with brown-seeded samples appearing in the yellow-seeded *S1* generation. Without an RFLP that marked the yellow-seeded homozygotes and the *Y* line samples, the RFLPs in the brown-seeded samples have limited use as selectable markers.
Ideally, a population from a controlled cross of parents, homozygous for the trait should have been used for the above study. Segregating patterns of the putative-RFLP marker for the seed colour trait could then be determined and the number of alleles involved in seed colour estimated. Although *B. carinata* accessions available at AAFC appear to be phenotypically diverse, the choice of parental germplasm would have to be carefully considered because DNA from these populations does not appear to be polymorphic (Appendix I and K); thus, detectable RFLP differences in the genomic DNA are uncommon.

5.4.1 Down-regulation of phenylpropanoid metabolism

The accumulation of *t*-cinnamic acid and the reduction of lignin content in the seed coats of *B. carinata Y* line suggest that phenylpropanoid metabolism was altered in yellow-seeded material. One source of altered metabolism may have arisen from altered DNA sequences in *CHS*. Although RFLPs were not exclusively associated with the B line, there were indications that differences related to seed colour existed in the *CHS* genomic DNA. This could produce alterations in either *CHS* transcripts or the catalytic behaviour of the *CHS* enzyme. Such alterations may be responsible for an accumulation of enzymatic precursors such as *t*-cinnamic acid in the yellow seed line. In keeping with reports that transcription of *CHS* can be down-regulated by *t*-cinnamic acid (>10^{-4} M) in alfalfa (*M. sativa*) protoplasts electroporated with a bean (*P. vulgaris*) *CHS* promoter sequence (Loake *et al*., 1991) and that phenylpropanoid accumulation in plant cells can be controlled at the level of substrate supply (Margna, 1977; Margna and Vainjarv, 1981), *t*-cinnamic acid levels could down-regulate *CHS* in yellow-seeded *B. carinata* and reduce overall flux through the flavonoid pathway.
Several lines of evidence in other plant systems point to PAL as an additional step which is potentially controlled by t-cinnamic acid and may contribute to the low concentration of lignin, condensed tannin and phlobaphene in yellow-seeded B. carinata if controlled in a similar fashion. Blount et al. (2000) have shown that when cinnamoyl-4-hydroxylase (C4H) activity was reduced in alfalfa by introducing an antisense C4H transgene, t-cinnamic acid levels increased and PAL activity was reduced. The converse (introduction of a PAL antisense transgene) did not affect C4H activity. Blount et al. (2000) proposed that flux in the phenylpropanoid pathway was controlled in part by feedback regulation mediated by C4H and t-cinnamic acid. Other investigators observed that a range of concentrations of t-cinnamic acid specifically down-regulated PAL activity and that subsequent flavonoid biosynthesis was reduced (Shields et al., 1982; Bolwell et al., 1986, 1988; Mavandad et al., 1990).

In the B. carinata Y line, the abundance of t-cinnamic acid in the seed coat extract was estimated from 2D-TLC separations to be ca. 25 µg (ca. 0.096 µmol per individual, mature seed coat). Although the estimated concentration of t-cinnamic acid was 10-fold lower in B. carinata than that shown to affect PAL by exogenous application in bean (Mavandad et al., 1990), the concentration of this substrate on a per-seed coat basis was calculated for mature B. carinata seed coat material. Whether this final concentration appeared earlier in the developing seed at the crucial time to influence enzyme activities is not known. Regardless, if it is accepted that the in vivo accumulation of t-cinnamic acid can specifically affect either transcription or enzyme activity, the in planta concentration of this metabolite observed for yellow-seeded B. carinata may have been effective in reducing the overall amount of flavonoids that
accumulated in seed coat tissue by feed-back inhibition of PAL and feed-forward inhibition of CHS.

It is speculative as to whether \( \tau \)-cinnamic acid contributed to regulating PAL or CHS and, ultimately, seed coat pigmentation and lignin synthesis in yellow-seeded \( B. \) carinata. Measurement of enzyme activities for PAL and other phenylpropanoid enzymes (e.g. C4H, 4CL) in \( Brassica \) seed coat material may help to determine whether feed-forward mechanisms are regulating these enzymes and contributing to differences in seed coat pigmentation and lignification. The impact of \( \tau \)-cinnamic acid on the total content of colourless flavonoids in yellow-seeded \( B. \) carinata or on enzymes involved in phenylpropanoid biosynthesis (PAL, C4H, 4CL) will not be known until experiments are conducted to measure \( \tau \)-cinnamic acid and these enzymes throughout seed development. Monitoring transcript levels of genes that encode enzymes such as C4H and 4CL could also contribute to understanding the effect of phenylpropanoid synthesis on the low lignin, unpigmented seed coat trait.

Although \( \tau \)-cinnamic acid may have contributed to reducing lignin concentration and down-regulating the flux of substrates to CHS for flavonoid biosynthesis, other products derived from phenylpropanoids did not appear to be affected. Esterified forms of sinapic acid (sinapine, sinapoyl methyl ester) were by far the most abundant compounds in the seed coat extracts of yellow- and brown-seeded \( B. \) carinata. Compounds such as sinapic acid and its methyl ester may represent hydrolysis products of sinapine. Sinapine was detected by TLC in unhydrolyzed crude seed coat extracts and did not appear to contribute to differences in seed coat colour. This compound is commonly found in \( Brassica \) seed extracts and is produced ubiquitously in most \( Brassica \) species (Fenton et al., 1980; Blair et al., 1984; Naczk et al., 1998).
5.5 Identification of phenylpropanoids and flavonoids by APcI LC-MS-MS

In the present study of seed coat pigment characteristics, several methods of separation and identification were used (solvent fractionation, cellulose TLC for preliminary characterizations by UV-illumination and UV-spectral shift reagents, analytical RP-HPLC using PDA detection and combined LC-MS techniques). In terms of definitive identification, separation by RP-HPLC with PDA detection for UV-spectra and elution into a MS for molecular mass determination were the most advantageous tools for identification of phenylpropanoids and flavonoids in seed coat extracts. The main advantage of LC-MS-MS was the increase in sensitivity using SIR to detect trace amounts of compounds suspected to be present in the extract (e.g. dihydromyricetin) and the added analysis of daughter ions in conjunction with in-source fragmentation patterns of the parent molecular ions.

Characteristic in-source fragmentation patterns were detected in the first analyzer from LC-separated extracts of seed coat aglycones and the components. [M+H]⁺ and [M-H]⁻, were observed. The coupling of RP-HPLC to MS-MS allowed aglycones to be identified by retention time, UV- and mass spectral information. The appearance of a molecular ion (in the NI or PI mode) from the sample extract at the same retention time as the authentic standard and with the correct mass was solid confirmation of the identity of the target compound. In-source fragmentation (LC-MS) of the standards and the unidentified compounds were the same and correlated with structure. The loss of a water molecule in dihydroquercetin from the parent molecular ion and the difficulty in fragmenting kaempferol, due to its series of conjugated bonds (without causing the molecule to completely disintegrate to masses less than 100 m/z) are examples of structure-related effects in the MS analyses.
Molecular ions were readily obtained from phenylpropanoid and flavonoid aglycones with soft ionization techniques such as APCI. However, some aglycones did not produce the expected positive molecular ion (\(\alpha\)-cinnamic acid, sinapic acid methyl ester) and in-source fragmentation was less evident from some \([M+H]^+\). In these cases, further tuning experiments to optimize mass detection for these specific compounds would resolve the difficulty. Optimal mass analyses would be useful in future flavonoid work to obtain quantitative analyses and molecular mass determinations comparable to surveys of other classes of natural products (He, 2000). The fragmentation patterns of some parent molecular ions from \(B. carinata\) seed coat extracts were more readily achieved in the second analyzer when the NI mode was used. This discovery was comparable to analysis of the \([M+H]^+\) and \([M-H]^-\) ions from flavonoid aglycones in a survey of the flavonoids from a member of the mint family (\(Ocimum gratissimum\) var. \(gratissimum\) L.) (Grayer et al., 2000). Daughter ions of the \([M-H]^-\) ions were preferentially analyzed in the \(O. gratissimum\) investigation rather than \([M+H]^+\) ions for the same reasons reported here for \(B. carinata\) analyses.

5.6 Research applications arising from the biochemical investigation of \(Brassica carinata\)

5.6.1 Phenolic fingerprinting of the \(Brassica\) A genome

Phenolic acids were examined in the unhydrolyzed MeOH extracts from whole seeds of several species of \(Brassica\) (section 4.6.3; Table 4.4, Figure 4.11). A distinctive group of UV-spectra characteristic of simple phenolic acids was present in those \(Brassica\) species that carry the A genome and was absent in species carrying only the B and/or C genomes. This “chemotaxonomic fingerprint” may be a useful profile in the same way marker-assisted selection is used, but in this case the \(Brassica\) A
genome would be detected rather than a single trait. Phenolic constituents as chemotaxonomic markers have been used in the past to characterize genera where species identification was difficult (Löffler et al., 1997; Williams et al., 1991) or where unethical food adulteration was suspected (Robards et al., 1997; Robards and Antolovich, 1995).

Although the chemotaxonomic marker for the A genome reported here was applicable to the accessions of Brassica species having the A genome, additional samples of several other cultivars should be analyzed in order to establish whether the occurrence of distinctive phenolic acids in the A genome is ubiquitous. Since environmental effects might have influenced the phenolic acid content in the original samples, the material for an expanded test should be grown concurrently so that the plants are subjected to identical field conditions. In the event that the phenolic acid profile reported here is due to specific alleles derived from a common ancestral A genome, it would be important to survey the A genome as widely as possible, including wild germplasm. Moreover, seed material should not be bulked and several samples from each population should be tested so as to ensure adequate sampling of all of the alleles in the population.

5.6.2 Anthocyanin assays as an indicator for the yellow seed trait

Leaves harvested from seedlings younger than the four leaf stage of the yellow-seeded line derived from the Y line of B. carinata had considerably reduced anthocyanin content compared to leaf tissue from the brown-seeded line, when the plants were grown in fluorescent light (VHO, Phillips) between 20 and 25°C. It was suggested previously (section 5.2.1) that the differences in the presence of anthocyanin in the Y line might be a pleiotropic effect arising from an altered DFR gene. Thus, leaf
anthocyanin content may serve as a rapid assay for selecting yellow-seeded material carrying this mutation from amongst a segregating population of *B. carinata* seedlings. If the iso-amyl extract procedure proves sensitive enough to detect anthocyanins from individual plants, then the assay could be used to track this trait in seedlings arising from interspecific crosses with the breeding lines of other *Brassica* germplasm. The change in visible leaf pigmentation in such seedlings may serve as a rapid, inexpensive method to determine the presence of the regulatory gene affecting *DFR*. As was outlined for the chemotaxonomic fingerprint test, sample size and growing conditions play an important role in determining the accuracy of the assay. It would be crucial not to induce anthocyanin biosynthesis by growing seedlings under very high intensity fluorescent light or at cool temperatures (15 to 18°C), since these conditions have been shown to induce anthocyanin biosynthesis (Kubasek *et al.*, 1992).

### 5.6.3 Impact of phlobaphenes and colourless phenolics on meal quality

Condensed tannins and phenolic acids (*e.g.* sinapine) reduce the nutritional quality of *Brassica* seed meals (Appendix C). Since phlobaphene and colourless copigmentation flavonoids in *B. carinata* seed coat tissue were shown here to be present in the yellow-seeded material, these compounds could also have a significant impact on seed meal quality. Phenolic complexes formed between condensed tannins and proteins, have been observed in seed meal-quality studies (reviewed in Naczk *et al.*, 1998). These complexes could just as likely occur with phlobaphenes, colourless flavonoids and phenolic acids, since the same type of covalent bonding and protein-phenolic interaction is theoretically possible. Although there is no documentation that discolouration by unoxidized, colourless phenolics occurred in canola meal during processing, there is a very real possibility that colourless phenolic acids and colourless
flavonoids in unhulled canola meal could darken during feed manufacture. Oxidation reactions were documented for the darkening of sinapic acid preparations that were exposed to air (Cai et al., 1999). These authors found that oxidized sinapic acid formed dark-coloured benzoquinone and napthoic acid end-products via a thomasidioic acid intermediate. The development of a yellow-seeded canola cultivar may assist in improving the oil concentration and reducing the fibre characteristics that currently interfere with meal quality using brown-seeded cultivars. However, it is equally important to evaluate the impact of colourless phenylpropanoids and flavonoid products on seed meal quality.

5.6.4 UV-fluorescent properties to distinguish sinapic acid and sinapine

Sinapic acid and its choline ester, sinapine, are antinutritional compounds that represent up to 90% of the total phenolic acid content in Brassica seed meal (Krygier et al., 1982a; Shahidi and Naczk, 1992; Pokorny and Réčlová, 1995; Simbaya et al., 1995; Matthäus, 1998). Sinapine (found only in the Brassicaceae) reduces the nutritional value of defatted canola meal for livestock feed, especially poultry (March and MacMillan, 1979; Butler et al., 1982b). Sinapic acid was shown to be converted to dark-coloured endproducts (Cai et al., 1999), which have the potential to create an unattractive, discoloured feed meal. Thus, strategies to lower the sinapine/sinapic acid content in canola-quality Brassica cultivars could have an economic benefit on canola seed meal with respect to competing in the marketplace with other feed ingredients such as soybean.

Plant storage compounds such as sinapoyl esters have long been the target of unsuccessful, concerted plant breeding efforts for elimination from germplasm devoted to canola meal improvement (Kräling et al., 1991). A reduction in sinapic acid and/or
sinapine might be achieved by tactics such as inhibiting sinapine synthase or incorporating the *sin-fahl* mutation found in an altered *Arabidopsis* which eliminated the accumulation of sinapic acid esters in seed tissues (Chapple *et al.*, 1992). The lack of heritable, genetic variation of low sinapine biotypes was cited as one of the reasons for the difficulty in eliminating sinapine from *B. napus* (Kräling *et al.*, 1991). In any case, a plant breeding program to reduce sinapine/sinapic acid in canola meal would require use of existing, accurate analytical methods in order to phenotype progeny that are low in sinapine.

Distinguishing sinapine from other ubiquitous simple phenolic acids (*e.g.* sinapic acid, caffeic acid, ferulic acid) commonly found in *Brassica* seeds is difficult, because most simple phenolic acids appear as fluorescent blue compounds under UV-illumination on cellulose TLC plates. These acids exhibit similar migration patterns on TLC plates and are not easily determined wholly by *R*<sub>f</sub> values from the complex mixtures characteristic of plant extracts (Table H.3). Although sinapic acid and sinapine standards exhibit very different *R*<sub>f</sub> values on cellulose plates developed in 15% HOAc, (*R*<sub>f</sub> 74 [sinapine] and *R*<sub>f</sub> 40 [sinapic acid]), these two compounds tend to behave unpredictably when present in a sample matrix. This altered mobility was evident in both preparative TLC and in unhydrolyzed MeOH extracts of whole seed separated by HPLC. Moreover, it is time-consuming and expensive to fully resolve the simple phenolic acids and their esters by RP-HPLC, because their retention times are very close, particularly when these compounds occur as esters as well (Table H.5, *e.g.* caffeoyl methyl ester, RT 14.58; sinapic acid, RT 15.27; ferulyl methyl ester, RT 15.35; ferulic acid, RT 15.36). Furthermore, the choline-ester moiety of sinapine does not absorb in the UV-wavelengths, so the UV-spectral properties of sinapine are practically
identical to those of sinapic acid. It may be an important advantage in breeding low-sinapine canola cultivars to be able to distinguish between sinapic acid and sinapine, because development of breeding lines may rely on genetic variation of the precursor(s) rather than sinapine, a storage product.

A precise method using cellulose TLC plates was discovered while analyzing sinapic acid and sinapine in B. carinata whole seed extracts. These compounds can be differentiated from other simple phenolic acids by observation on cellulose TLC plates: sinapic acid is turquoise-green under UV-light with NH₃ fuming and fades to a distinctive purple-blue, whereas sinapine is yellow-green under UV-light with NH₃ fuming and fades to a distinctive turquoise-blue. Other simple phenolic acids do not share these properties under NH₃ fuming. This feature provides a simple and relatively fast method to determine the sinapine/sinapic acid content in Brassicaceae material. Quantification is not as precise as HPLC analysis although relative estimations are possible by keeping the seed sample small (100 mg) and using equalivant solvent-extraction volumes in comparative assays.

Several authors reported unidentified phenolic acid-like compounds in TLC and gas chromatographic analyses of canola/rapeseed meal (reviewed in Naczka et al., 1998; Fenton et al., 1980). The authors speculated that these unknowns were methyl esters of phenolic acids (ferulic, p-coumaric, sinapic), possibly with forms of cis-trans isomerism. However, since authentic standards of phenolic esters are not commercially available, the novel detection method described here to identify sinapine/sinapic acid could be useful for identifying the two major phenolic acids in canola seed meal. Since the method is an inexpensive assay requiring little technical expertise, it could be used where elaborate analytical facilities are unavailable.
CHAPTER 6

CONCLUSIONS

The research reported in this dissertation showed that three phenolic polymers were reduced or missing in the seed coat of yellow-seeded B. carinata: lignin, condensed tannin, and phlobaphene. Instead, colourless phenylpropanoids (e.g. t-cinnamic acid) and flavonoids (dihydroflavonols and flavonols) accumulated in the seed coat of the yellow-seeded line. By comparison, the lignin concentration was higher, condensed tannins and phlobaphenes were heavily deposited, and the metabolic intermediates were absent in the seed coats of the brown-seeded material (either cv S-67 or the B line). In addition, there was possibly a pleiotropic effect on pigment synthesis in the Y line plants because minimal anthocyanin was present in seedling tissue grown at 20 to 25°C compared to tissue of the B line.

The lack of condensed tannins and phlobaphenes in seed coat tissue of B. carinata Y line may be due to changes that affect DFR expression, because end products beyond the DFR enzymatic step in flavonoid synthesis were not formed. Analysis of DFR expression in developing seed by RT-PCR confirmed that the abundance of DFR transcripts was lower in yellow-seeded B. carinata, which was consistent with the accumulation of dihydroflavonols and flavonols in mature seed coats. Furthermore, lowered lignin concentration implied that there was a change in the expression or activity of at least one of the phenylpropanoid enzymes in the yellow-seeded line. Trans-cinnamic acid may have a regulatory effect on synthesis in the
phenylpropanoid pathway. One possible change could have occurred at the C4H step because reduced lignin content was associated with alterations in this enzyme instead of PAL, the entry point to the phenylpropanoid pathway according to reports in studies of transgenic Arabidopsis and tobacco (Sewalt et al., 1997). Measurement of PAL, C4H and 4CL transcripts in yellow-seeded and brown-seeded B. carinata might clarify the contribution of these genes to pigment deposition and lignin concentration.

A number of regulatory proteins for controlling DFR expression in developing seed or the pericarp have been reported in other genera. These regulatory proteins may be good candidates for investigating control of DFR expression in B. carinata. Proteins encoded from loci such as AtMYC1 in Arabidopsis, rk in red kidney bean, An family of loci in petunia and C1-I in maize (Paz-Ares et al., 1990; Quattrocchio et al., 1993; Urao et al., 1996; Albert et al., 1997; Beninger and Hosfield, 1999) are prospects for DFR regulation both in B. carinata and other Brassica species. Of the loci suggested here, only the C1-I and BAN alleles have been reported to exhibit a dominant form of inheritance.

The inheritance of alleles in interspecific crosses is unpredictable because it can be difficult to foretell how the genomes may recombine. When yellow-seeded B. carinata was crossed with cv Westar, a dark-seeded B. napus, the seed colour of the F1 progeny was black (Rashid et al., 1994). In this cross, there may be more than one regulatory factor affecting flavonoid metabolism that contributed to down-regulating the expression of seed coat pigmentation in B. carinata (BBCC genomes), and these proteins appear to have behaved differently in the B. napus genetic background (AACC). Conversely, the B. napus used in the cross (cv Westar) may be carrying an unknown regulatory factor that was otherwise missing or silent in B. carinata. The
scope of genetic control of the enzymes controlling seed coat pigmentation could be quite extensive based on the pigment patterns (hilum, scattered spots and mottling) and types of pigments reported here. Development of a set of mutants similar to the mutants in Arabidopsis (e.g. tt, ast) would be helpful in characterizing Brassicaceae seed coat genotypes.

In this research, the chemical nature of the seed coat pigments was investigated in a small sample of Brassicaceae species. Otherwise, the work focused on the chemical constituents and gene expression of DFR with respect to the yellow seed trait in B. carinata. Considering the recessive nature of yellow seed inheritance in B. rapa, B. napus and B. juncea, in-depth analyses of these species might produce different results. Preliminary evidence (TLC and RP-HPLC-MS) from seed coat extracts of a yellow-seeded line of B. juncea showed a high concentration of flavonols (relative to the B. carinata Y and B line extracts) and a similar accumulation of t-cinnamic acid and dihydroquercetin. Although the flavonoid composition of a corresponding near-isogenic line of brown-seeded B. juncea was not tested for comparison, this initial data provided reason to believe that a similar regulator was exerting control on flavonoid endproduct synthesis by affecting the expression of DFR.

Seed coat pigmentation comparisons between near-isogenic yellow- and brown-seeded lines could be undertaken to determine the nature of the phenolics in seed coat tissue from other agriculturally important mustard- and oil-seed crops. The complexity of phenylpropanoid and flavonoid metabolism in yellow-seeded germplasm that was demonstrated in this present research of B. carinata contributes to explaining some of the sources of genetic control of pigment biosynthesis and ultimately, to developing higher quality, yellow-seeded canola cultivars of B. napus.
Investigation of such complex regulation in a pathway is illustrative of the post-genomics era. Plant genomes such as rice and _Arabidopsis_ are completely sequenced and a plethora of sequences for previously unknown proteins and regulatory factors in plants has been discovered. The next step is to elucidate the function of these proteins as part of the metabolic profile. The evidence for complex metabolic controls at key points in a biosynthetic pathway such as found for _DFR_ is illustrative of the importance for assigning functions to these newly-revealed gene sequences. Plant biochemistry stands on the brink of exciting discoveries related to gene regulation and plant metabolism. In understanding the genetic controls on gene expression, new insights into metabolic signaling cascades, not only between enzymatic complexes and cell organelles, but also between the plant and the environment, may be accomplished.
Chapter 7

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APPENDICES
APPENDIX A. The ‘Triangle of U’: the genomic relationships of certain Brassicaceae

\[ \begin{align*}
B. \text{nigra} \\
& \text{BB} \\
& n = 8 \\
\end{align*} \]

\[ \begin{align*}
B. \text{carinata} \\
& \text{BB CC} \\
& n = 17 \\
\end{align*} \]

\[ \begin{align*}
B. \text{juncea} \\
& \text{AA BB} \\
& n = 18 \\
\end{align*} \]

\[ \begin{align*}
B. \text{oleracea} \\
& \text{CC} \\
& n = 9 \\
\end{align*} \]

\[ \begin{align*}
B. \text{napus} \\
& \text{AA CC} \\
& n = 19 \\
\end{align*} \]

\[ \begin{align*}
B. \text{rapa} \\
& \text{AA} \\
& n = 10 \\
\end{align*} \]

Adapted from U, 1935
Appendix B Evaluation of methods for analysis of plant polyphenols

Methods to isolate and characterize phenolics differ in their ability to discriminate for the particular compound under investigation. This evaluation was written to address the suitability of commonly-used methods and to provide background information for selecting one method in preference to another for the research presented in this dissertation.

B.1 Isolation and detection of condensed tannins

B.1.1 Accuracy and precision of the vanillin assay

Numerous assays have been developed for detecting condensed tannin polymer. One of the earliest assays was the vanillin assay (Bate-Smith and Lerner, 1954). Some controversy surrounds the use of assays such as vanillin because of the difficulty in determining how many oligomeric tannin units are measured. This concern has led to evaluation of the factors that influence the assay (extraction time, background sample colour, assay temperature, catechin as a reference compound, relative concentration of vanillin to HCl) (Deshpande et al., 1989; Price et al., 1978). The vanillin assay does not discriminate on the basis of molecular mass (i.e. degree of polymerization) and overestimates condensed tannins if there is a pool of catechins, dihydrochalcones or leucoanthocyanidins present (Price et al., 1978; Sarkar and Howarth, 1976). Later research determined that vanillin detects flavan-3-ol end groups, although the precise reaction with a tannin polymer is not described (Butler et al., 1982a).

Several studies resolved questions about the precision of the vanillin assay. Polyphenols with meta-oriented -OH groups on the A ring undergo substitution reactions very easily, thereby facilitating the formation of an adduct with vanillin at one or the other of these positions (Figure B.1) (Deshpande et al., 1989; Deshpande and
Condensation reaction of vanillin with leucoanthocyanidin.

In acidic solutions, vanillin is converted to vanillic acid and can react at unsubstituted C6 (illustrated) and/or C8(*) positions on the A ring when there is no carbonyl group at position 4(†) on the C ring. (adapted from Deshpande et al., 1989).
Salunkhe, 1982). These substitutions occur on the A ring at C-6 (or less often, at C-8) when there is no carbonyl group (C = O) at C-4 on the C ring. Therefore, flavan-3-ols form these type of adducts with vanillin very readily and stain a bright red. Flavonols (e.g. quercetin) do not react with vanillin because the C = O deactivates the A ring even though the A ring hydroxylation pattern contains meta-oriented -OH groups. At no time does the B ring enter into adduct formation (Mole and Waterman, 1987; Deshpande and Salunkhe, 1982). Once the vanillic radical forms the intermediate adduct, a dehydration reaction occurs and a red-coloured condensation product forms (a characteristic positive). Thus catechin and leucoanthocyanidins give a strong positive response because they are very reactive monomers. Condensed tannins are polymers with fewer reactive positions and give a lower response unless the polymer is acid-hydrolyzed to cleave flavan-3-ols. This is a source of underestimation in condensed tannin analyses because it is difficult to determine the degree of polymerization remaining after the cleavage reaction (Salunkhe et al., 1990; Deshpande et al., 1989).

The selection of a reference standard for assays that depend on colour development is most accurate when the standard is based on the partial purification of the polymer from the source plant family itself (Mole and Waterman, 1987). The reaction kinetics, such as linearity and reaction times, are different in condensed tannin assays with tannins from different plant sources (Mole and Waterman, 1987; Price et al., 1978). Some authors suggest that dissimilar mixtures of monomeric and polymeric flavans are present in extracts from different plant sources (Salunkhe et al., 1990; Deshpande et al., 1989; Deshpande and Salunkhe, 1982).

Vanillin assay conditions and reagents have been thoroughly examined. Precision and accuracy of the vanillin assay was optimized in sorghum by a vanillin
reaction with catechins and by a vanillin reaction with purified condensed tannins. It was discovered that the type of acid (acetic, hydrochloric, sulfuric), reaction time, reaction temperature, vanillin concentration and reagent freshness, water concentration in the vanillin reagent, presence of interfering substances in the sample, and the standard utilized are all important factors (Sun et al., 1998). This is true for both a vanillin reaction with catechins or a vanillin reaction with oligomeric units such as condensed tannins. The vanillin assay is more precise with glacial HOAc than with MeOH (Butler et al., 1982a). However, since the kinetics of the two types of reactions are markedly different, it is recommended that reaction times and with different plant sources be individually optimized (Sun et al., 1998; Terrill et al., 1992).

**B.1.2 Prussian Blue assay**

The Prussian Blue reaction for measuring condensed tannins is based on the reduction of ferric ions (Fe$^{3+}$) by tannin moieties (and also any other phenolic compound) from K$_3$Fe[CN]$_6$ reagent to a ferrous product (Fe$^{2+}$) by the formation of a ferricyanide-ferrous complex (Price and Butler, 1977). This blue-coloured complex absorbs maximally at 720 nm; low concentrations of condensed tannins are perceived as shades of green. The precision of the assay is very sensitive to the use of organic solvents in the extract (such as MeOH) and to the amount of unreacted ferricyanide. Since the Prussian Blue reaction is sensitive to all polyphenols, it is not appropriate in crops such as rapeseed/canola since it detects other phenolics, such as sinapine. It is very suitable, however, for use in minimal scientific facilities such as a grain elevator when crops other than canola are analyzed (Price and Butler, 1977). Prussian Blue is used as a diagnostic spray for polyphenols on TLC plates (Conde et al., 1992). This is
a useful method to discriminate between polyphenolic substances and other non-polar compounds present in methanolic plant extracts.

Price and Butler (1977) compared the Prussian Blue assay to the vanillin assay by using catechin (a flavan-3-ol) as a standard. The vanillin assay was considered less useful because, in the Prussian Blue assay, concentration is estimated by a range of colour from green to blue, whereas the vanillin assay was uniformly red over the same concentration range. Price and Butler (1977) suggest that the Prussian Blue assay is also more accurate because vanillin over-estimates the tannins (based on catechin equivalents).

B.1.3 BuOH-HCl assay

The BuOH-HCl assay overcomes the difficulty of measuring a variably-sized polymer such as condensed tannins (Kristiansen, 1986; Porter et al., 1986). Butanol-HCl is a very efficient solvent for hydrolyzing the condensed tannin polymer to monomers ('anthocyanidins'). The condensed tannins are depolymerized in strong acidic solutions to yield anthocyanidin pigments, which are quantified by a spectrophotometer at 550 nm (Watterson and Butler, 1983). To remove the effect of other compounds which absorb at 550 nm, the condensed tannin can be first preferentially bound to polyvinylpolypyrrolidone (PVPP). The PVPP-condensed tannin complex is washed with MeOH-HCl to remove non-bound material. Subsequently, condensed tannin polymers are cleaved by hydrolysis in BuOH-HCl to form anthocyanidins, an autoxidation reaction in which the yield is dependent on trace metal-ion impurities (Porter et al., 1986). Iron (Fe^{3+}) salts in the reaction medium improved the analytical precision although other research results demonstrated that the iron could interfere with reproducibility (Terrill et al., 1992). The bound condensed
tannin units are released from the PVPP by heating in BuOH-HCl. Absorption at 550 nm is measured by a wavelength scan between 400 and 700 nm; interpolation of the underlying curve not part of the anthocyanidin-specific peak at 550 nm is subtracted (Skadhauge et al., 1997). This is an accurate and linear determination of condensed tannins that addressed the problem of specifically estimating the concentration of complex condensed tannin polymers and eliminating compounds such as anthocyanins with a similar absorbance (Carron et al., 1994; Terrill et al., 1992). The BuOH-HCl assay is a complementary to the vanillin assay. The vanillin assay measures only the terminal flavan-unit because a simple adduct to the tannin oligomer is formed without disrupting the polymer (Porter et al., 1986). The BuOH-HCl treatment hydrolyzes the condensed tannin polymer and provides a measurement of these cleavage products, rather than forming adducts with terminal units (Porter et al., 1986).

If PVPP is used in vanillin reactions to remove interfering compounds with a similar absorbance, the adduct created by the vanillin reaction remains bound to the PVPP and its absorbance is not readily measured. Condensed tannin is also underestimated in Prussian Blue tests with PVPP (Watterson and Butler, 1983). Only 25% as much colour forms with PVPP-bound material compared to colour that develops in an assay solution without PVPP. These authors concluded that condensed tannin assays with PVPP are best confined to the BuOH-HCl hydrolysis method.

**B.2 Isolation and detection of the lignin fraction**

**B. 2.1 Crude and dietary fibre: traditional methods to estimate lignin**

The traditional methods to estimate lignin concentration have relied on weighing an insoluble residue. The established terms ‘crude fibre’ and ‘dietary fibre’
describe digestibility factors, rather than a compound-specific designation such as 'lignin', 'cellulose' or 'hemicellulose'.

Crude fibre analysis is based on the relative weight of plant material after severe extraction with petroleum ether, strong acid and then alkali. The extraction recovers such highly variable amounts of cellulose (50-80%), lignin (10-50%), and up to 20% hemicellulose, that the values are not very useful (Yoshida and Kuwano, 1989). An acid-insoluble fraction ('Klason' lignin) is measured by weighing the acid-insoluble residue after ignition in a muffle furnace at 500°C for 2 hours to drive off carbohydrate material (Theander et al., 1977). This is an improvement on estimates of lignin by crude fibre values because the carbohydrate component is removed. Nutritionists have replaced crude fibre determinations by 'dietary fibre' analysis, defined as "plant parts that are resistant to hydrolysis by human digestive enzymes" (Spiller, 1986). Dietary fibre represents a fraction that consists largely of lignin but also includes cellulose components, and is considered superior to 'crude fibre' for evaluating nutritive values for monogastric livestock (Yoshida and Kuwano, 1989; Spiller, 1986).

Improvements in fibre determinations were made when samples were treated to remove some of the non-fibrous constituents. These extractions use sulfuric acid and cetyl trimethylammonium bromide (CTAB) (a detergent) to solubilize proteins (van Soest, 1963). Lignin and associated polyphenols are determined by the calculation "Lignin = (N.D.F. - [N.S.P. + protein + ash])" where N.D.F. is neutral detergent fibre and N.S.P. is detergent-soluble, non-starch polysaccharides (Simbaya et al., 1995; van Soest, 1963).

Rapeseed meal fibre was estimated by a titration method, a further improvement in the Klason analysis (Stringam et al., 1974). Defatted meal and hulls were extracted
first in mild concentrations of sulfuric acid then in potassium hydroxide to remove proteins. The remaining pellet was treated with acidified chromate potassium iodide for a colorimetric reaction with Na$_2$S$_2$O$_3$. The fibre was estimated precisely by titration with Na$_2$S$_2$O$_3$ and starch to a blue-green endpoint. This colorimetric method is a more specific determination of fibre concentration and was previously used to measure fibre in *B. carinata* (Getinet, 1986).

### B.2.2 Estimation of thioglycolic lignin

The thioglycolic lignin method (thioacetolysis) which involves the preparation of partially-purified, alkali-soluble lignin monomers is an improvement on the foregoing procedures (Whitmore, 1978a; 1978b). A hot methanol incubation removes unbound phenolic acids and esters from the plant material. This is followed by treatment of the tissue with thioglycolic-HCl to hydrolyze complex plant polymers (*e.g.* cellulose, lignin, cell-wall polysaccharides) and to release membrane- and cell wall-associated proteins. Acid precipitation preferentially removes the lignin monomers (monolignols) from the solution (Hammerschmidt, 1984). Subsequently, monolignols are re-solubilized in 1 M NaOH to provide a suitable extract on which to measure absorbance (Hammerschmidt, 1984; Whitmore, 1978a).
Appendix C  Phenolic compounds that affect canola meal quality

'Quality' in seed meal refers to the ability of the meal to nourish livestock with little or no nutritional loss due to indigestibility (Bell, 1993). Good quality feed meal should be free of ingredients that interfere with the absorption of important nutritional components such as protein and phosphorus (Naczk et al., 1998). Components in canola meal, such as phytate (a six carbon sugar with bound phosphates) and phenolics present in seed coat pigments (e.g. condensed tannins) or as co-pigments, react with protein and create bound products that are nutritionally unavailable to livestock (Simbaya et al., 1995; Sarwar et al., 1981). Canola meal is high in fibre as well as simple and complex phenolics. These indigestible and antinutritional components have been implicated in reduced weight gains in livestock and poultry (Bell, 1993; Bell and Shires, 1982; Butler et al., 1982b).

C.1  Fibre: an indigestible component that lowers canola meal quality

When the fibre content in livestock feed is too high, the feed is less digestible; hence, not all of the metabolizable energy is available to livestock such as poultry and monogastrics. Moreover, high fibre content in livestock feed creates problems for the producer because more manure is produced, creating a waste management problem as well as the need for more feed to be supplied to maintain weight gains, especially in swine at the young weaner stage (Dr. B. Roosnagel, University of Saskatchewan, pers. communication).

The hull fraction (seed coats) in canola meal is the source of most of the indigestible components for monogastrics. A high proportion of these compounds was attributed to the fibre content of canola hulls because this material is ca. 16% of the seed weight and ca. 30% of the meal weight (Bell, 1993; Bell and Shires, 1982; Butler
These indigestible substances are derived from fibre and cell-wall bound phenolic acids *(e.g. ferulic acid, caffeic acid)* (Matthäus, 1998; Naczk *et al*., 1998; Bell, 1993; Shahidi and Naczk, 1992; Kozlowska *et al*., 1990; Mitaru *et al*., 1982; Theander *et al*., 1977; Durkee, 1971). These compounds were present in lower amounts in yellow-seeded brassicas with unpigmented hulls such as 'Yellow Sarson' *(B. rapa)* and white mustard *(Sinapis alba)* (Bell, 1993; Kozlowska *et al*., 1990; Kozlowska *et al*., 1983; Theander *et al*., 1977) compared to the dark-seeded brassicas, *B. nigra, B. rapa* cv Echo and *B. napus* cv Tower (Shirzadegan and Röbbelen, 1985; Krygier *et al*., 1982a; Krygier *et al*., 1982b; Durkee and Thivierge, 1975; Stringam *et al*., 1974; Durkee, 1971). Phenolic moieties detected as ‘bound’ were derived from fibre-associated polymers, such as lignin. ‘Fibre’ consists mainly of lignin, a cell wall-associated phenolic polymer, but also includes complex polysaccharides such as cellulose and hemicellulose (Bell, 1993; Mitaru *et al*., 1982; Stringam *et al*., 1974). Stringam *et al* (1974) predicted that a 7% to 11% reduction in fibre content would expand canola meal usage in animal feed and that versatility of the meal would be enhanced by using yellow-seeded cultivars due to the non-speckled, light appearance of the meal. This would permit greater flexibility in feed formulation without changing the appearance of the product.

There are conflicting reports in the literature regarding both the composition and the amount of bound phenolic acids in crude fibre of canola meal. The bound fraction is a small proportion of the total phenolic acids of rapeseed and it is difficult to determine such low quantities accurately (Shahidi and Naczk, 1992). Environmental differences likely play a role in determining the final concentration of phenolic acids and tannin-related compounds in seed. Other seed quality parameters *(e.g. fibre,
protein and oil content; the degree of pigmentation mottling the yellow-seeded
cultivars) in several *Brassica* species have been demonstrated to vary from one field
season to the next (Naczk *et al.*, 1998; Getinet *et al.*, 1996; Rashid *et al.*, 1994; Bell,

C.2 Effect of soluble phenolics on canola meal quality

The predominant soluble phenolic components in canola meal are
phenylpropanoids. The main constituent (which accounts for 91% of the phenolics) is
sinapic acid and its esterified form, sinapine (Shahidi and Naczk, 1992; Kozlowska *et
al.*, 1990; Blair and Reichert, 1984; Fenton *et al.* 1980). In addition, other
antinutritional substances occur as chemical artifacts of the feed processing operation.
Phenolics (*e.g.* sinapine) oxidize or react with other meal components to produce dark-
coloured substances during the heating required to remove solvent residues left over
from oil extraction (Shahidi and Naczk, 1992). Phenolics or their oxidation products
interact with meal proteins and inhibit digestive enzymes (*e.g.* oxidases, lipases,
peptidases). The oxidized breakdown products of the phenolics are particularly
reactive because they form covalent bonds with the carbonyl groups in proteins to
create indigestible complexes (Cai *et al.*, 1999; van Sumere *et al.*, 1975; van Buren and
Robinson, 1969). Lysine- and methionine-rich peptides are nutritionally unavailable to
monogastric digestive systems because condensed tannin breakdown products bind to
the amino or thioether group of these amino acids (Riberéau-Gayon, 1972). Ortho-
quinones form from dihydroxy phenolics (*e.g.* caffeic acid) by phenol oxidase catalysis
and polymerize non-enzymatically with aminothiol groups in proteins. These
polyphenolic complexes have been linked to growth depression in chicks and ducklings
as well as 'egg-taint' in brown-egg laying poultry (Bell, 1993; Bell and Shires, 1982;
Butler et al., 1982b). The bioavailability to laboratory rats of a wide range of essential minerals was affected by polyphenols. In particular, inhibition of iron absorption by tannin-rich materials was reported (Nyman and Björck, 1989). In total, oxidized phenolics bound up to a third of the dry weight of proteins in canola meal (Sosulski, 1979), and together with decreased absorption of essential minerals, could indicate the reason for growth depression in poultry. Seed meal processed experimentally with only yellow-seeded cultivars reduced both anti-nutritional complexes and fibre, thereby increasing the nutritional value of canola meal (Matthäus, 1997; Simbaya et al., 1995; Slominski and Campbell, 1991; Shirzadegan and Röbbelen, 1985; Butler et al., 1982b).
Appendix D  Plant propagation

D.1  Development of yellow- and brown-seeded lines of *B. carinata* in growth-chamber and greenhouse facilities

In 1995, fifty-six seeds of the yellow-seeded *B. carinata* accession PGRC/E 21164 were planted, one seed to a pot, in 20-cm pots in a soiless mix (Appendix D.7). These were placed in a controlled-environment growth-chamber (Conviron, Winnipeg, Canada), under fluorescent and incandescent, high-intensity lighting (photosynthetically-active radiation readings [320 to 510 µE], Licor Model LI-185B), with an 18 h photoperiod at 22°C, reduced to 20°C during dark time. The plants were re-arranged on the benches in the growth-chamber twice monthly to ameliorate the effects of the lower light intensity (320 µE) at the ends of the light banks as well as other possible environmental gradients (*e.g.* temperature, air-flow). The plants were individually bagged (perforated cellophane crossing bags [CryoVac, Winnipeg, Canada]) for selfing when the floral meristem had emerged. Mature seed (*S*₁ seed) was threshed individually from each plant, 5 to 6 months after the planting date. Subsequent generations were developed as shown, Figure 3.1 (section 3.1) except as follows: one month after the yellow-seeded *S*₂ generation had germinated, the plants were moved from the growth-chamber to a greenhouse with natural lighting (mid-summer to late autumn) and 18 to 20°C day/15°C night temperatures until maturity. Subsequently, another growth-chamber became available and all further plant material was grown in similar conditions to the *S*₁ plants, unless otherwise described.

D.2  Source of plant material used for restriction enzyme digests

Ten plants from thirty-six individuals of the *B. carinata* PGRC/E 21164 *S*₁ generation (used to develop the Y and B line material) and ten plants of the Y and B
lines were grown in the greenhouse in the soiless mix, five plants per 20-cm pot. (These ten plants were bulked at harvest). Natural light was supplemented by high pressure sodium lamps equipped with Philips 430 Agros light bulbs to extend the photoperiod to 18 h in 18/15°C day/night conditions. All the leaf tissue was harvested (stems and leaf mid-ribs discarded) when the plants reached the five- to six-leaf stage. Leaves were harvested into perforated, cellophane crossing bags (CryoVac, Winnipeg, Canada), immediately frozen in liquid nitrogen, freeze-dried, then stored at −20°C.

D.3 Source of tissue for gene expression studies of developing seed

The tissue for monitoring gene transcripts was harvested from *B. carinata* PGRC/E 21164 Y and B plants. The seeds were planted, one to a pot, and grown in the greenhouse under fluorescent light banks (VHO bulbs [Phillips], supplemented with 100 watt incandescent light [GE]) with a 18 h photoperiod at 22°C, reduced to 20°C during dark time. When flowering began, unopened buds were emasculated and then self-pollinated using pollen from opened flowers on the same plant. The racemes were checked frequently and opened flowers removed in order to prevent pollen from circulating in the growth-chamber. Controlled pollination was performed in this way on five or six racemes per plant, then all further floral meristems were removed. Each pollination event was tagged so that ‘days after pollination’ (dap) were known. All plants were kept bagged (perforated cellophane crossing bags) to prevent accidental cross-pollination if new flowers opened and were not removed immediately.

Developing seed was hand-harvested from the siliques 5, 10, 20 and 30 dap. In addition, two other tissue types were collected: (1) the apices of young floral meristems were collected 62 days after planting; (2) unopened buds suitable for pollination.
designated ‘0 dap’. All plant material was immediately frozen in liquid nitrogen as soon as it was dissected and then stored at −80°C.

D.4 Source of seedling leaf tissue for anthocyanin determination and RNA

Seedlings of B. carinata PGRC/E 21164, Y and B lines were grown for leaf tissue in the greenhouse as described in D.2. Another set of plants were grown under similar illumination but at cooler temperatures (18°C, reduced to 15°C during dark time). Leaves for anthocyanin determinations were harvested at the 2 to 3 leaf stage, ca. 12 d after germination and extracted immediately for anthocyanins (section 3.6).

For RNA extractions, 200-mg samples from the plants described above were harvested. Four leaf stages were collected starting ca. 8 d after germination: (1) ‘C’, cotyledons fully developed; (2) ‘1’, first leaf to emerge and open; (3) ‘2’, second leaf to open, third leaf beginning to emerge; (4) ‘3’ third leaf opened: fourth leaf visible. Total RNA extracts from this leaf tissue provided a positive control on Northern blots for gene transcripts of the phenylpropanoid and flavonoid genes and for the comparison of DFR expression between warm- and cool-grown seedlings.

D.5 Production of field-grown B. carinata from S₁, S₂, and S₃ generation seeds to test the seed coat pigmentation trait

Once the PGRC/E 21164 Y and B lines were produced, the expression of the seed coat pigmentation trait was tested under field conditions. Ten seeds from PGRC/E 21164 Y line generations S₁, S₂, and S₃ and ten seeds from the S₁ and S₂ PGRC/E 21164 B line generations were planted in a replicated single block design in the AAFC B. carinata nursery plots in May, 1998. AC Excel (a black-seeded B. napus) was planted as a check for the development of seed coat pigments.

D.6 Source of additional Brassicaceae seed used for phenolic profiles

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The *B. carinata* cultivar S-67, genetically different from the *B. carinata* PGRC/E 21164 B line germplasm, was also used for determining phenolics in brown-seeded material. Seeds of S-67 were taken from 1990 bulked seed of a Canadian-grown tent increase (open-pollinated). This field-grown (f.g.) material was used to examine seed coat pigmentation in the histochemical tests, assays for lignin and extractable condensed tannins, and the initial extractions for phenolic profiles. In addition, at the beginning of this research project, samples of cv S-67 were grown in the same growth-chamber as the S₁ generation of *B. carinata* PGRC/E 21164 Y line. Plants were kept bagged to prevent cross-pollination. Seed from these cv S-67 plants were designated “cv S-67 growth-chamber” (g.c.). This material was described with these terms in assays where both these samples were used. If not so described, the material was from only the f.g. source.

Seed of other Brassicaceae (*B. juncea* cv Lethbridge 22A, *B. juncea* cv AC Vulcan, *B. juncea* ‘Common Brown’, *B. juncea* cv Blaze, *B. juncea* ‘ZYR-6’ , *B. napus* germplasm [described below], *B. nigra* SRS 192 (Type 2), *B. oleracea* SRS 652, *B. oleracea* SRS 2400, *B. rapa* cv AC Parkland, *B. rapa* cv Echo, *Crambe abyssinica* [Hochst. ex O.E. Schulz] cv Prophet, *Eruca sativa* [Mill.] SRS 2681. *Raphanus sativus*, *Sinapis alba* cv Ochre, *S. alba* SRS 2495 and *S. arvensis* [L.]) was used for seed coat histochemistry, extractable condensed tannin and lignin analyses. Field grown seed from bulked samples was provided by either Mr. Don Rode (AAFC, Saskatoon, Canada) or by Mr. Richard Gugel (Plant Gene Resources, Saskatoon, Canada). The F₅ progeny (prefixed by ‘KF95’) from a cross between *B. napus* YN90-1016 (mottled, yellow-seeded) and Westar (dark brown-seeded) were provided by Mr. Ken Friesen (AAFC, Saskatoon, Canada) for determinations of lignin and extractable condensed
tannin concentration. Fresh (≤ 1 month) A. thaliana cv Columbia seed was provided from material grown in the AAFC greenhouses. Arabidopsis seeds were used only in the histochemical detection of anthocyanins and condensed tannins.

D.7 Recipe for soilless mix for Brassica species plantings

Developed by Mr. W.H. Leonard, Greenhouse Manager, AAFC, 1960-1980

Sphagnum peat moss 107 L
Medium grade vermiculite 110 L
Calcium carbonate, fine ground 3.2 kg
‘Osmocote Plus’ slow-release fertilizer (16-8-12) plus minor nutrients 3.5 kg
Superphosphate, 20 %, fines (0-20-0) 700 g
Trace elements #555 (Frit Industries Inc., Ozark, AL) 15.0 g
Iron chelate, 13 % 15.0 g
Zinc chelate, 14 % 7.0 g

Sand, washed and screened to 1 mm maximum particle size added as 1 part to 4 parts total volume; final range of pH 5-6 adjusted by amount of calcium carbonate.

Appendix E Mechanical collection of B. carinata seed coat (hulls)

Brassica carinata whole seed samples that were enriched for the seed coat fraction were prepared using the Tangential Abrasive Dehulling Device (TADD) (Oomah et al., 1979). Seed samples (300 g) from both Y and B lines yielded 76 to 78 g of seed coat tissue including ca. 10% cotyledon tissue. A grinding plate with 120 grit abrasive paper was rotated (ca. 1000 rpm) underneath the seeds in bursts of 1 min intervals for 5 to 6 min. Automatic vacuum aspiration collected the seed coat material by venturi action.
Appendix F  Reagents and probes for DNA extraction and analyses in Southern
and Northern hybridizations

Solutions were mixed with sterile, HPLC-grade water.

F.1  ‘Kirby Mix’ for high-quality DNA extraction (Covey and Hull, 1981)

Tri-isopropylnaphthalene-sulphonic acid, sodium salt (Acros)  1 g
4-Aminosalicylic acid (Sigma)  7.25 g
2M Tris-HCl, pH 8.0 (Sigma)  2.5 mL
Buffer-saturated phenol (lower layer)  6 mL
Water  90 mL

F.2.  Source of sequences and preparation of radioactive probes

Cloned DNA sequences were used to provide molecular probes for radioactive
($^{32}$P-dCTP) labeling. A sample of each plasmid was sent for sequencing (Plant
Biotechnology Institute [PBI], Saskatoon, Canada). The nucleotide sequences were
compared to the database in ‘Blastn’ (Altschul et al., 1990; Pearson et al., 1988) to
discover the identity of the insert.

◊  CHS

A genomic CHS clone was amplified from B. napus by PCR with primers
designed from the S. alba CHS sequence (containing the first intron and the second
exon), cloned as a 1200 bp insert in a plasmid (Bluescript II KS [Stratagene]) (gift of
Dr. I. Parkin, AAFC, Saskatoon, Canada). Plasmids were purified from the host E.
coli, strain DH5-α, with a commercial kit (Wizard Plus, Promega) according to the
manufacturer’s instructions. The insert in the plasmid was digested with BamH1 and
Sal1 and separated on a 0.8% agarose gel in order to purify the probe (Prep-a-Gene,
BioRad) for radioactive labeling.

◊  PAL

A PAL clone, CD3-122 (Accession No. L33678) was obtained from the
Arabidopsis Biological Resource Centre (Columbus, OH). The A. thaliana PAL
sequence was a 300 bp insert contained in Bluescript SK(+) and with homology to the
5’ region of PAL2 transcripts (Wanner et al., 1995). The deduced PAL2 amino acid
sequence exhibits 90% similarity to most dicot PAL sequences (Wanner et al., 1995).
The probe was prepared by using M13 primers (sequence, Appendix G) to amplify the
300 bp insert by PCR. This produced a 535 bp fragment because the primers anneal on either side of the multiple cloning site in the plasmid to include ca. 235 bp extra.

- **CAD**

Primers were designed from a published *A. thaliana* sequence (Baucher et al., 1995) to amplify a 300 bp fragment from the second exon of *CAD* in *B. carinata* by PCR (primer sequence, Appendix G). The PCR amplification (program, Appendix F.3.4) product included 300 bp fragments that were ligated into the plasmid 'pGEM-T' (Promega) following manufacturer's directions and cloned into *E. coli* (DH5-α) by electroporation (Bio-Rad), and grown on selective media according to Sambrook et al. (1989). The *CAD* probe was prepared from a purified insert with a confirmed *CAD* sequence and used for hybridization.

- **DFR**

Initial RT-PCR reactions used *A. thaliana* primers for *DFR* (Appendix F.3.2, G.3) and the reverse transcriptase-generated cDNA from total RNA extracts, as the template. A 400 bp fragment was generated in every sample of cDNA from seedlings and developing seed of *B. carinata* Y and B lines. Several cDNA clones were constructed by ligating excised, purified fragments (Prep-a-Gene [BioRad]) into the plasmid 'pGEM-T' (Promega) following manufacturer's directions. These plasmids were cloned into *E. coli* (DH5-α) by electroporation and grown on selective media. The cloned inserts were submitted to PBI for sequencing and verified for sequence homolgy using the NCBI database (Altschul et al., 1990; Pearson et al. 1988) (Figure F.1).

- **Actin**

Actin is a constitutively produced transcript in all plant tissues; thus, it is frequently used to indicate the amount of RNA loaded for each sample in the gel and as an estimate of the integrity of the RNA preparation. The forward and reverse primers for actin and a 1.5 kb fragment amplified from *B. napus* and ligated into Bluescript SK(-) plasmid were obtained from Dr. P. Bonham-Smith, Department of Biology, University of Saskatchewan (primer sequence, Appendix G). This clone is listed with NCBI as AF111812 (1998).
AJ251982.1|ATH251982 *Arabidopsis thaliana* *DFR* gene for
dihydroflavonol 4-reductase,
Exons 1-6, wild type allele
Length = 1831

Query: 76   aatttgaagaaagtcagcatctctttgatttgcacaacgcgaacgcaactcaactttta 135
                  [ ] [ ] [ ] [ ] [ ] [ ] [ ] [ ] [ ] [ ] [ ] [ ] [ ] [ ] [ ] [ ] [ ] [ ] [ ] [ ] [ ] [ ]
Subjct: 382 aatttgaagaagtaacataactctctttgatttgcacaacgcgaacgcaactcaactttta 441

Query: 136 tggaaagccgatttattctgacgaaggaagctacgtagctacgctataacggatgcgacggc 195
                  [ ] [ ] [ ] [ ] [ ] [ ] [ ] [ ] [ ] [ ] [ ] [ ] [ ] [ ] [ ] [ ] [ ] [ ] [ ] [ ] [ ] [ ]
Subjct: 442 tggaaagctgattttttatctgaggaaggaagctacgtagctacgctataacggatgcggt 501

Query: 196 gtttcccacatagcaacctcccatggattttgatctcaaggatc 239
                  [ ] [ ] [ ] [ ] [ ] [ ] [ ] [ ] [ ] [ ] [ ] [ ] [ ] [ ] [ ] [ ] [ ] [ ] [ ] [ ] [ ] [ ]
Subjct: 502 gtttcccagctgcaacaccatggattttgatctcaaggatc 545

Figure F.1 Sequence comparison of a cloned *DFR* fragment (400 bp) from a RT-PCR
reaction using *B. carinata* cDNA as the template and a published *DFR*
sequence of *Arabidopsis thaliana*. Comparison made using NCBI
‘Blastn’ database program with the Fasta format (Altschul *et al.*, 1990;
F.3 Amplification of cDNA in RT-PCR

F.3.1 PCR reaction buffer for RT-PCR (final concentration 50 μL reaction⁻¹)

10x Tris (pH 8.0), 1x
100 mM KCl, 20 mM
25 mM MgCl₂, 2.5 mM
2 mM dNTPs, 0.2 mM (each nucleotide)
5 μM primer (forward and reverse), 0.4 μM or 0.6 μM
Taq polymerase (5U μL⁻¹), 1U
cDNA template ca. 25 ng

F.3.2 Thermal cycling program for RT-PCR using primers based on DFR sequences in A. thaliana (Appendix G.3).

<table>
<thead>
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<th>Step</th>
<th>Temp (°C)</th>
<th>Time (min)</th>
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<td>94</td>
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<td>0:35</td>
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<tr>
<td>4</td>
<td>72</td>
<td>1:00</td>
</tr>
</tbody>
</table>
Repeat steps 2 to 4, for 28 cycles
| 5    | 72        | 7:00       |
| 6    | 4         | forever    |

F.3.3 Thermal cycling program for RT-PCR using primers based on DFR sequences in A. thaliana and B. carinata (Appendix G.4).

<table>
<thead>
<tr>
<th>Step</th>
<th>Temp (°C)</th>
<th>Time (min)</th>
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<tr>
<td>1</td>
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<td>4</td>
<td>72</td>
<td>0:30</td>
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Repeat steps 2 to 4, for 38 cycles
| 5    | 72        | 7:00       |
| 6    | 8         | forever    |
F.3.4 Thermal cycling program for amplification of genomic DNA to create a CAD probe.

<table>
<thead>
<tr>
<th>Step</th>
<th>Temp (°C)</th>
<th>Time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>94</td>
<td>1:00</td>
</tr>
<tr>
<td>2</td>
<td>94</td>
<td>1:00</td>
</tr>
<tr>
<td>3</td>
<td>42</td>
<td>1:00</td>
</tr>
<tr>
<td>4</td>
<td>72</td>
<td>1:00</td>
</tr>
</tbody>
</table>

Go to step 2, for 5 cycles

<table>
<thead>
<tr>
<th>Step</th>
<th>Temp (°C)</th>
<th>Time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>94</td>
<td>1:00</td>
</tr>
<tr>
<td>7</td>
<td>50</td>
<td>1:00</td>
</tr>
<tr>
<td>8</td>
<td>72</td>
<td>1:00</td>
</tr>
</tbody>
</table>

Repeat steps 5 to 8 for 30 cycles

<table>
<thead>
<tr>
<th>Step</th>
<th>Temp (°C)</th>
<th>Time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>9</td>
<td>72</td>
<td>7:00</td>
</tr>
<tr>
<td>10</td>
<td>4</td>
<td>forever</td>
</tr>
</tbody>
</table>
Appendix G  Primer sequences

G.1 CAD primers, based on a published A. thaliana sequence
Forward primer: 5’ AGG CTG CAC CGC TAC TCT G 3’  (T_m = 53°C)
Reverse primer: 5’ CAA GTA AGG GTC GAG AGG GTG 3’  (T_m = 53°C)
Primer synthesis gift of Dr. G. Scoles, Molecular Genetics Lab, Department of Plant Sciences, University of Saskatchewan, Saskatoon, Canada

G.2 M13 primers
Universal primer  5’ TGT AAA ACG ACG GCC AGT 3’
Reverse primer  5’ CAG GAA ACA GCT ATG AC 3’
Primers gift from Mr. D. Schwab, PBI Sequencing Lab, Saskatoon, Canada

G.3 DFR primers, based on published (NCBI) A. thaliana sequences within the third exon. These primers produce a 400 bp fragment related to DFR sequences from B. carinata mRNA or genomic DNA and was cloned and sequenced for subsequent use as a probe (Appendix F.2)
Forward 5’ CAA AAA TGG TTA GTC AGA AAG AGA 3’  (T_m = 50°C)
Reverse 5’ CTA AGC ATC GGT TCT CTC GCC 3’  (T_m = 51°C)

G.4 DFR primers, for RT-PCR experiments, expected to produce a 206 bp fragment. Forward primer is based on the first exon of a published A. thaliana sequence for DFR; reverse primer (3’) is based on a B. carinata sequence obtained from sequencing a cloned fragment of a PCR reaction with primers in G.3 and B. carinata genomic DNA. These primers produce a 200 bp fragment from B. carinata cDNA.
Forward 5’ CTT CGG GTT TCA TCG GTT CAT 3’  (T_m = 51°C)
Reverse 5’ TCC GTT TAT AGC GTC ATC GTA GC 3’  (T_m = 55°C)

G.5 Actin primers (amplify a ca. 650 bp fragment in Brassica spp.)
Forward primer 5’ CCC TGC CAT GTA TGT TGC 3’  (T_m = 67°C)
Reverse primer 5’ GCC AAG ATG GAT CCT CC 3’  (T_m = 68°C)
Primers are a gift from Dr. P. Bonham-Smith, Department of Biology, University of Saskatchewan, Saskatoon, Canada.
Appendix H  Analytical separations of authentic standards

H.1  Silica gel TLC

Four different solvent systems were evaluated for the ability to resolve a mixture of standards on silica gel plates (Table H.1, one solvent system per plate) to obtain an indication of the class of phenylpropanoids and flavonoids present in *Brassica* species. Migration rates were generally very fast (<30 min) and the order of migration was very consistent even though absolute R_f values were inconsistent. No single mobile phase completely resolved all the classes of flavonoids; however, the best resolution was achieved with the mobile phase 45% EtOAc in CHCl_3 (Table H.1; Figure H.1). It was also evident that the migration of faster moving components affected the slower migrating compounds. A very non-polar phenylpropanoid (3,4-dimethoxycinnamate), spread out to overlap and increase the migration of the slower moving flavonol, myricetin. When a mixture of five standards that included 3,4-dimethoxycinnamate and myricetin was developed with EtOAc: formic acid: water (66:14:20) (Amarowicz *et al.*, 1995) on silica TLC, the lane with the standards resolved into only three discrete spots and myricetin was not evident (Figure H.1, A).
Table H.1  One-dimensional separation characteristics of phenylpropanoids and flavonoids on silica gel TLC plates in four different solvent systems.

<table>
<thead>
<tr>
<th>Solvent Mobile Phase</th>
<th>Phenolic acids</th>
<th>Phenolic ester</th>
<th>Chalcone</th>
<th>Flavone</th>
<th>Flavanone</th>
<th>Flavonol</th>
<th>Dihydroflavonol</th>
<th>Glycoside</th>
<th>Solvent Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHCl₃ : MeOH 93 : 7</td>
<td>H</td>
<td>L</td>
<td>M-H</td>
<td>H</td>
<td>H</td>
<td>M</td>
<td>M-H</td>
<td>n.t.²</td>
<td>Condé et al 1992</td>
</tr>
<tr>
<td>45% EtOAc in CHCl₃</td>
<td>L to M</td>
<td>n.t.</td>
<td>H</td>
<td>L⁺, L²</td>
<td>L⁺, M</td>
<td>L</td>
<td>M</td>
<td>n.t.</td>
<td>Marles</td>
</tr>
</tbody>
</table>

*Mobility rating: H, high; M, medium; M-H, medium high; L, low*

¹glycosides, refers to flavonoids that are not aglycones, thus are water-soluble

²n.t., not tested

³Research scientist, AAFC, Saskatoon, Canada (personal communication)

⁴mobility when hydroxylation present on B-ring
Figure H.1 Comparison of two mobile phases to separate phenylpropanoid and flavonoid standards on silica 1D-TLC plates.

(A) Separation of standards developed with EtOAc: formic acid: water (66: 14: 20) (Amarowicz et al., 1995).
(B) Separation of authentic standards using 45% EtOAc in CHCl₃.

Identification of standards:
(↑) acacetin (flavone), (↑) myricetin, (flavonol), (↓) 3,4-dimethoxycinnamate (phenolic acid), (↑) 5-hydroxy flavone,
(↓) 5,7-dihydroxyflavone, (M) mixture of standards
H.2 Cellulose TLC

Seven solvent systems were evaluated for their ability to resolve a mixture of flavonoid and phenylpropanoid standards on microcrystalline cellulose-coated 1D-TLC plates (Table H.2). Cellulose TLC is best for more polar compounds (e.g. phenolic acids, hydroxylated flavonoids, flavonols). Forestal and BAW require longer development times (>3 h) compared to the solvent systems for silica plates (ca. 25 min). Cellulose TLC complements the fast, non-polar separation features characteristic of silica gel TLC.

Closely-related compounds, such as myricetin, quercetin and kaempferol, are difficult to separate completely under some conditions using 1D cellulose chromatography (Table H.2, H.3). In a TLC separation developed with a highly polar solvent such as 15% acetic acid or Forestal, these compounds resolve in the order ‘myricetin, quercetin and kaempferol’ when measured from the origin. However, in the less polar solvent, BAW, quercetin and kaempferol do not completely resolve and produce one elliptical spot which can interfere with observing other compounds migrating closely. The $R_f$ values for a series of standards on cellulose plates (Table H.3) provide a general guide to identifying unknowns and to demonstrate relative migration differences between classes of phenylpropanoids and flavonoids. Two-dimensional TLC experiments with a mixture of standards and later, seed extracts, were conducted using two different concentrations of HOAc (15%, 30%) and Forestal or BAW. These results determined the method chosen to separate seed coat extracts by 2D-TLC.
Table H.2  One-dimensional separation characteristics of phenylpropanoids and flavonoids on cellulose TLC plates in seven different solvent systems.

<table>
<thead>
<tr>
<th>Mobile Phase</th>
<th>Phenolic acids</th>
<th>Phenolic ester</th>
<th>Chalcone</th>
<th>Flavone</th>
<th>Flavanone</th>
<th>Flavanol</th>
<th>Dihydroflavanol</th>
<th>Glycoside&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Solvent Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>BAW&lt;sup&gt;2&lt;/sup&gt;</td>
<td>M-H</td>
<td>M-L</td>
<td>H</td>
<td>H</td>
<td>M-H</td>
<td>M-H</td>
<td>M-H</td>
<td>M-L</td>
<td>Harborne, 1984</td>
</tr>
<tr>
<td>Water</td>
<td>L to M</td>
<td>M</td>
<td>L</td>
<td>L</td>
<td>L</td>
<td>L</td>
<td>L</td>
<td>H</td>
<td>Onyilagha, 1994</td>
</tr>
<tr>
<td>15% HOAc</td>
<td>M to H</td>
<td>H</td>
<td>M-L</td>
<td>v. L</td>
<td>v. L</td>
<td>M</td>
<td>H</td>
<td></td>
<td>Mabry &lt;i&gt;et al.&lt;/i&gt;, 1970</td>
</tr>
<tr>
<td>50% HOAc</td>
<td>L</td>
<td>L</td>
<td>n.t.</td>
<td>3M-H</td>
<td>3M-H</td>
<td>M-L</td>
<td>L</td>
<td></td>
<td>Mabry &lt;i&gt;et al.&lt;/i&gt;, 1970</td>
</tr>
<tr>
<td>CAW&lt;sup&gt;4&lt;/sup&gt;</td>
<td>M-H</td>
<td>M</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>M-H</td>
<td>Mabry &lt;i&gt;et al.&lt;/i&gt;, 1970</td>
</tr>
<tr>
<td>Forestal&lt;sup&gt;5&lt;/sup&gt;</td>
<td>H</td>
<td>M</td>
<td>M</td>
<td>H</td>
<td>M-H</td>
<td>M-L</td>
<td>M to M-H</td>
<td>n.t.</td>
<td>Harborne, 1984</td>
</tr>
</tbody>
</table>

<sup>1</sup>glycosides' refers to flavonoids that are not aglycones, thus are water-soluble

<sup>2</sup>Butanol : HOAc : water (4:1:5)

<sup>3</sup>Except methylated compounds, which barely separated from origin

<sup>4</sup>CHCl<sub>3</sub> : HOAc : water (30:15:2)

<sup>5</sup>conc. HCl : HOAc : water (3:30:10)

<i>Mobility rating: very low (v. L); low (L); medium-low (M-L); medium (M); medium-high (M-H); high (H); not tested (n.t.)</i>
Table H.3  Relative mobility of authentic standards separated by cellulose TLC.

<table>
<thead>
<tr>
<th>Compound</th>
<th>R&lt;sub&gt;f&lt;/sub&gt;§</th>
<th>15%</th>
<th>30%HOAc</th>
<th>BAW</th>
<th>Forestal</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Yellow/orange/gold-brown</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>acacetin (5,7-dihydroxy,4-methoxyflavone)</td>
<td>1</td>
<td>-</td>
<td>90</td>
<td>87</td>
<td></td>
</tr>
<tr>
<td>5,7-dihydroxyflavone</td>
<td>3</td>
<td>-</td>
<td>90</td>
<td>72</td>
<td></td>
</tr>
<tr>
<td>eriodictyol (3',4',5,7-tetrahydroxyflavanone)</td>
<td>7</td>
<td>44</td>
<td>90</td>
<td>83</td>
<td></td>
</tr>
<tr>
<td>5-hydroxyflavone</td>
<td>19</td>
<td>-</td>
<td>88</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>fisetin (flavonol)</td>
<td>9</td>
<td>14</td>
<td>58</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>kaempferol (flavonol)</td>
<td>7</td>
<td>11</td>
<td>56</td>
<td>51</td>
<td></td>
</tr>
<tr>
<td>quercetin (flavonol)</td>
<td>6</td>
<td>7</td>
<td>55</td>
<td>32</td>
<td></td>
</tr>
<tr>
<td>myricetin (flavonol)</td>
<td>5</td>
<td>7</td>
<td>47</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>sakuranetin (flavanone)</td>
<td>6</td>
<td>-</td>
<td>88</td>
<td>90</td>
<td></td>
</tr>
<tr>
<td><strong>Fluorescent blue/light blue</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>caffeic acid (phenolic acid)</td>
<td>21</td>
<td>-</td>
<td>77</td>
<td>69</td>
<td></td>
</tr>
<tr>
<td>3,4-dimethoxycinnamic acid (phenolic acid)</td>
<td>48</td>
<td>65</td>
<td>90</td>
<td>85</td>
<td></td>
</tr>
<tr>
<td>sinapic acid (phenolic acid)</td>
<td>40</td>
<td>33</td>
<td>78</td>
<td>82</td>
<td></td>
</tr>
<tr>
<td>sinapine (choline ester of a phenolic acid)</td>
<td>74</td>
<td>-</td>
<td>60</td>
<td>96</td>
<td></td>
</tr>
<tr>
<td>sinapyl alcohol (blue-green component)</td>
<td>-</td>
<td>50</td>
<td>76</td>
<td>91</td>
<td></td>
</tr>
<tr>
<td><strong>Pale/very light blue</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7,4'-dihydroxyflavone</td>
<td>11</td>
<td>-</td>
<td>82</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td><strong>Deep purple-blue or black</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dihydrokaempferol (dihydroflavanol)</td>
<td>45</td>
<td>58</td>
<td>-</td>
<td>87</td>
<td></td>
</tr>
<tr>
<td>dihydroquercetin (dihydroflavanol)</td>
<td>-</td>
<td>42</td>
<td>-</td>
<td>80</td>
<td></td>
</tr>
<tr>
<td>dihydromyricetin (dihydroflavanol)</td>
<td>-</td>
<td>40</td>
<td>-</td>
<td>70</td>
<td></td>
</tr>
<tr>
<td>luteolin (3',4',5,7-tetrahydroxyflavone)</td>
<td>3</td>
<td>-</td>
<td>80</td>
<td>77</td>
<td></td>
</tr>
<tr>
<td>naringenin chalcone</td>
<td>22</td>
<td>-</td>
<td>86</td>
<td>56</td>
<td></td>
</tr>
<tr>
<td>phloretin chalcone</td>
<td>20</td>
<td>-</td>
<td>85</td>
<td>56</td>
<td></td>
</tr>
<tr>
<td><strong>Invisible in λ&lt;sub&gt;366&lt;/sub&gt;</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>trans-cinnamic acid (phenylpropanoid)</td>
<td>54</td>
<td>70</td>
<td>90</td>
<td>88</td>
<td></td>
</tr>
</tbody>
</table>

§ R<sub>f</sub> x 100 (ratio of the distance the compound travels to the distance the solvent front travels). Theoretically, when the conditions of measurement are completely specified, the R<sub>f</sub> value is constant for any given compound because it corresponds to a physical property of that compound. Values are averaged from two consecutive experiments; “-” not determined.
The appearance of the standards under UV-illumination, with and without ammonia fuming is distinctive for certain classes of flavonoids, particularly when combined with the relative position on cellulose TLC plates (Table H.4). Characteristic spray compounds can distinguish classes of flavonoids. Vanillin has been reported to stain flavan-3-ols red (as well as flavan-3,4-diole and dihydrochalcones, e.g. phloretin) (Sarkar and Howarth., 1976). Naturstoffe reagent has been reported to distinguish between flavones and flavonols with different hydroxylation patterns (Sheahan and Rechnitz, 1993; Wagner et al., 1984). This should be a useful reagent because some flavanone, flavone and flavonol standards display the same yellow or fluorescent yellow-orange colour under UV-illumination (Table H.4) and fuming with NH₃ does not discriminate further between compounds in these classes. However, Naturstoffe reagent did not behave in the manner indicated in the literature with kaempferol (4’, 5, 7-trihydroxy-flavonol) and luteolin (3’, 4’, 5, 7-dihydroxyflavone). Instead, Naturstoffe caused these flavonols and flavones to fluoresce orange (regardless of the hydroxylation pattern) while flavanones remained yellow. Nonetheless, this differentiation permitted the flavanones to be distinguished from the flavones and flavonols. This could be due to the type of TLC plate and the presence or absence of binders for the coating or fluorescent indicators.
Table H.4  Appearance of authentic standards separated on cellulose 2D-TLC under UV-illumination. Solvents: (1) 15% or 30% HOAc (2) Forestal.

<table>
<thead>
<tr>
<th>Colour¹ under UV (λ_{366})</th>
<th>Colour under UV (λ_{366})/NH₃ ⁴</th>
<th>Colour under UV(λ_{280})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yellow/orange/gold-brown</td>
<td>Brightens/becomes golden</td>
<td>Not visible</td>
</tr>
<tr>
<td>acacetin (flavone)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5,7-dihydroxyflavone</td>
<td></td>
<td></td>
</tr>
<tr>
<td>eriodictyol (flavanone)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-hydroxyflavone</td>
<td></td>
<td></td>
</tr>
<tr>
<td>fisetin (flavonol)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>kaempferol (flavonol)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>myricetin (flavonol)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>quercetin (flavonol)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>sakuranetin (flavanone)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fluorescent blue/light blue</td>
<td>Most turn a shade of darker blue</td>
<td>Not visible</td>
</tr>
<tr>
<td>caffeic acid (phenolic acid)</td>
<td>yellow-blue</td>
<td></td>
</tr>
<tr>
<td>3,4-dimethoxycinnamic acid</td>
<td>dark blue</td>
<td></td>
</tr>
<tr>
<td>4-hydroxycinnamic acid</td>
<td>light blue</td>
<td></td>
</tr>
<tr>
<td>ferulic acid (phenolic acid)</td>
<td>blue</td>
<td></td>
</tr>
<tr>
<td>sinapic acid (phenolic acid)</td>
<td>turquoise green\longrightarrow fades to a distinctive purple-blue</td>
<td></td>
</tr>
<tr>
<td>sinapine (esterified phenolic)</td>
<td>yellow-green\longrightarrow fades to a distinctive turquoise blue</td>
<td></td>
</tr>
<tr>
<td>Pale/very light blue</td>
<td>Bright blue/light blue</td>
<td>Not visible</td>
</tr>
<tr>
<td>7,4'-dihydroxyflavone</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Deep purple-blue or black</td>
<td>Most turn purple-black</td>
<td>Not visible</td>
</tr>
<tr>
<td>dihydrokaempferol (dihydroflavanol)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>dihydromyricetin (dihydroflavanol)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>dihydroquercetin (dihydroflavanol)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>luteolin (flavone)</td>
<td>paler</td>
<td></td>
</tr>
<tr>
<td>naringenin chalcone</td>
<td>orange</td>
<td></td>
</tr>
<tr>
<td>phloretin chalcone</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Invisible in λ_{366} irrespective of fuming trans-cinnamic acid (phenolic acid)  Visible black

¹ colour after fuming with NH₃ and immediately illuminating with UV λ_{366}
⁴ Colours differ slightly when plate is developed in BAW instead of Forestal.
Specific observations are recorded for each compound only where different from general class.
### H.3 Analytical RP-HPLC

Table H.5 | UV-spectral library of phenylpropanoid and flavonoid standards separated by RP on a Symmetry C\textsubscript{18} column on HPLC ‘B’ (section 3.3.1) using the 33-min gradient.

<table>
<thead>
<tr>
<th>Compound name</th>
<th>Phenolic Class</th>
<th>RT (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{p}-coumaric glucoside</td>
<td>phenolic acid</td>
<td>4.09</td>
</tr>
<tr>
<td>3,5-dihydroxybenzoate</td>
<td>phenolic acid</td>
<td>4.18</td>
</tr>
<tr>
<td>4-O-ferulic glucoside, ME\textsuperscript{3}</td>
<td>esterified phenolic acid</td>
<td>5.26</td>
</tr>
<tr>
<td>chlorogenic acid</td>
<td>esterified phenolic acid</td>
<td>7.99</td>
</tr>
<tr>
<td>2,5-dihydroxybenzoate</td>
<td>phenolic acid</td>
<td>9.72</td>
</tr>
<tr>
<td>caffeic acid</td>
<td>phenolic acid</td>
<td>10.87</td>
</tr>
<tr>
<td>sinapine</td>
<td>esterified phenolic acid</td>
<td>12.10 (14.74)</td>
</tr>
<tr>
<td>dihydromyricetin</td>
<td>dihydroflavanol</td>
<td>12.54 (14.72)</td>
</tr>
<tr>
<td>\textit{p}-coumaric acid</td>
<td>phenolic acid</td>
<td>14.22</td>
</tr>
<tr>
<td>caffeoyl methyl ester</td>
<td>esterified phenolic acid</td>
<td>14.58</td>
</tr>
<tr>
<td>sinapic acid</td>
<td>phenolic acid</td>
<td>15.27 (23.20)</td>
</tr>
<tr>
<td>feruoyl methyl ester</td>
<td>esterified phenolic acid</td>
<td>15.35</td>
</tr>
<tr>
<td>ferulic acid</td>
<td>phenolic acid</td>
<td>15.36</td>
</tr>
<tr>
<td>dihydroquercetin</td>
<td>dihydroflavanol</td>
<td>16.04 (24.94)</td>
</tr>
<tr>
<td>fisetin</td>
<td>flavonol</td>
<td>18.17</td>
</tr>
<tr>
<td>myricetin</td>
<td>flavonol</td>
<td>18.19 (33.02)</td>
</tr>
<tr>
<td>dihydrokaempferol</td>
<td>dihydroflavanol</td>
<td>18.28 (31.00)</td>
</tr>
<tr>
<td>7.4'-dihydroxyflavone</td>
<td>flavone</td>
<td>19.10</td>
</tr>
<tr>
<td>3,4-dimethoxycinnamic acid</td>
<td>phenolic acid</td>
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</tr>
<tr>
<td>eriodictyol</td>
<td>flavanone</td>
<td>20.70</td>
</tr>
<tr>
<td>quercetin</td>
<td>flavonol</td>
<td>20.98 (40.12)</td>
</tr>
<tr>
<td>\textit{t}-cinnamic acid</td>
<td>phenolic acid</td>
<td>21.95 (44.59)</td>
</tr>
<tr>
<td>sakuranetin</td>
<td>flavanone</td>
<td>22.05</td>
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<tr>
<td>3-methoxycinnamic acid</td>
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<td>dihydrochalcone</td>
<td>23.09</td>
</tr>
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<td>chalcone</td>
<td>23.14</td>
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<tr>
<td>kaempferol</td>
<td>flavonol</td>
<td>23.51 (49.97)</td>
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<td>flavone</td>
<td>23.63</td>
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<tr>
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<td>flavone</td>
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<td>luteolin</td>
<td>flavone</td>
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<tr>
<td>acacetin</td>
<td>flavone</td>
<td>27.37</td>
</tr>
</tbody>
</table>

\(^1\) RT with the 55-min gradient method in brackets; \(^2\) methoxy ester
H.4 Extracted ion chromatograms and mass spectra for selected phenylpropanoids and flavonoids in Y line seed coat extract.

Total ion current chromatograms (TIC) were produced for the Y and B line seed coat extracts (Figure H.2-7, A) (data for B line not shown). Extracted ion current chromatograms were then generated for each ion corresponding to the [M-H]$^-$ ion for the compounds of interest (t-cinnamic acid, the dihydroflavanols, quercetin and kaempferol) (Figures H.2-7, B). In additional injections on the LC-MS-MS, the mass analyzer was adjusted to monitor only a selected m/z (single ion recording [SIR]). In this way, only the ions at the chosen m/z are detected. This equipment configuration significantly increased the instrument’s sensitivity so that trace quantities not apparent in the TIC could be detected. The LC-MS-MS quadrupole was then tuned to detect only the selected m/z corresponding to the ions previously examined (by selected ion monitoring), and the samples re-injected (e.g. to detect dihydromyricetin) (Figure H2, B). The mass spectrum of the peak was displayed when a scan of the selected m/z produced a detectable peak at the correct retention time (Figures H.2-7, C). The ‘correct’ retention time was determined by subsequent LC-MS-MS injections using authentic standards. The mass spectrum of each unidentified compound (eluting at the same time and with the same m/z as the standard) was compared to the mass spectrum of the authentic standard to confirm the identification (Figures H.2-7, D).
Figure H.2  LC-MS-MS scans (SIR, negative ion mode) and mass spectrum of dihydromyricetin (DHM) in the Y line, 100% MeOH fraction.
(§ total number of molecular ions detected; *retention time)

A. Chromatogram of the total ion current, showing molecular ions [M-H]⁻ in the Y line sample that were detected by the mass analyzer.

B. Selected ion recording of the Y line sample showing that the [M-H]⁻ with a m/z of 319 (DHM, 320 da) coincides with the peak at the same retention time as the DHM standard.

C. Mass spectrum of DHM in sample.

D. Mass spectrum of authentic DHM standard.
Figure H.3  LC-MS-MS scans (negative ion mode) and mass spectrum of dihydroquercetin (DHQ) in the Y line, 100% MeOH fraction.

(§ total number of molecular ions detected; *retention time)

A. Chromatogram of the Total Ion Current, showing molecular ions [M-H]– in the Y line sample that were detected by the mass analyzer.

B. Extracted Ion Chromatogram of the Y line sample showing that the [M-H]– with a m/z of 303 (DHQ, 304 da) coincides with the peak at the same *retention time as the DHQ standard.

C. Mass spectrum of DHQ in sample; D. mass spectrum of authentic DHQ standard.
Figure H.4  LC-MS-MS scans (negative ion mode) and mass spectrum of dihydrokaempferol (DHK) in the Y line, 100% MeOH fraction.

($) total number of molecular ions detected; *retention time

A. Chromatogram of the total ion current, showing molecular ions [M-H]− in the Y line sample that were detected by the mass analyzer.

B. Extracted ion chromatogram of the Y line sample showing that the [M-H]− with a m/z of 287 (DHK, 288 da) coincides with the peak at the same *retention time as the DHK standard.

C. Mass spectrum of DHK in sample.

D. Mass spectrum of authentic DHK standard.
Figure H.5  LC-MS-MS scans (negative ion mode) and mass spectrum of t-cinnamic acid in the Y line, 100% MeOH fraction.

(§ total number of molecular ions detected; * retention time)

A. Chromatogram of the total ion current, showing molecular ions [M-H]⁻ in the Y line sample that were detected by the mass analyzer.

B. Extracted ion chromatogram of the Y line sample showing that the [M-H]⁻ with a m/z of 147 (t-cinnamic acid, 148 da) coincides with the peak at the same *retention time as the t-cinnamic acid standard.

C. Mass spectrum of t-cinnamic acid in sample.

D. Mass spectrum of authentic t-cinnamic acid standard.
Figure H.6 LC-MS-MS scans (negative ion mode) and mass spectrum of quercetin in the Y line, 100% MeOH fraction.

(§ total number of molecular ions detected; *retention time)

A. Chromatogram of the total ion current, showing molecular ions [M-H]⁻ in the Y line sample that were detected by the mass analyzer.

B. Extracted ion chromatogram of the Y line sample showing that the [M-H]⁻ with a m/z of 301 (quercetin, 302 da) coincides with the peak at the same *retention time as the quercetin standard.

C. Mass spectrum of quercetin in sample.

D. Mass spectrum of authentic quercetin standard.
Figure H.7  LC-MS-MS scans (negative ion mode) and mass spectrum of kaempferol in the Y line, 100% MeOH fraction.

(5 total number of molecular ions detected; *retention time)

A. Chromatogram of the total ion current, showing molecular ions [M-H]^- in the Y line sample that were detected by the mass analyzer.

B. Extracted ion chromatogram of the Y line sample showing that the [M-H]^- with a m/z of 285 (kaempferol, 286 da) coincides with the peak at the same *retention time as the kaempferol standard.

C. Mass spectrum of kaempferol in sample.

D. Mass spectrum of authentic kaempferol standard.
Appendix I  Marker-assisted selection using randomly amplified DNA

I.1  Introduction

Analysis of DNA is useful for plant breeders because desirable traits can be monitored in a segregating population by using a DNA sequence associated with that trait ( Tanksley, 1993; Tanksley et al., 1989). Such DNA sequences may indicate the presence of traits for which it is difficult to select at the seedling stage (e.g. seed coat pigmentation) or because it is a quantitative trait (e.g. yield). Hence, DNA sequences that are used to select traits in this way are known as “molecular markers”. This is particularly useful for quantitative traits controlled by several loci. Molecular markers for such traits can be generated by judicious use of the polymerase chain reaction (PCR) with a primer (10 to 12 nucleotides [nt]) of random DNA sequence (Welsh and McClelland, 1990; Williams et al., 1990). The primer arbitrarily anneals to homologous DNA sequences throughout the genome; hence the names “randomly amplified” or “arbitrarily primed” are used to describe these DNA polymorphisms (Welsh and McClelland, 1990).

I.2  Bulked segregant analysis

While RAPD polymorphisms could be mapped in a segregating population and linked to the trait of interest, most RAPD markers have been identified using bulked segregant analysis (BSA). One bulk is a pooled DNA sample constructed from the DNA samples of plants that carry a trait. A second bulk is prepared from DNA of several plants that do not carry the trait. To be effective, the plants used to constitute the bulks should derive from a segregating population and thus have a common genetic background. Ideally, the progeny that are used to create the pooled DNA bulks are homozygous for the trait undergoing the analysis. Thus, a polymorphism that is
present between the parents and present in one of the bulks corresponding to the phenotype of the parental DNA is considered a potential molecular marker. For example, BSA was recently used to find several polymorphisms correlated with the high/low erucic acid trait in *S. alba* (Drost, 1999). Eleven percent of the random primers tested produced polymorphisms between the bulked DNA samples that were associated with the erucic acid concentration.

Besides progeny from a conventional cross, doubled-haploid (DH) populations can also be used in BSA. The identification of molecular markers associated with seed colour in *B. napus* used BSA and a DH population derived from a cross of *B. napus*, YN90-1016 x Apollo (Somers *et al.*, 1997). Both sample bulks and each parent of the original cross (one of which will bear the desired phenotype) were used for the template DNA in PCR reactions. Eight RAPD markers were identified that clustered in an interval 21.6 cM around a locus associated with seed colour (Somers *et al.*, 1997).

I.3 Bulked segregant analysis of a segregating population of *B. carinata*

I.3.1 Origin of tissue for BSA

Seeds (four each) from the Y and B lines were grown as parents for reciprocal crosses and 150 progeny propagated to the F$_3$ generation in the green house under the same conditions as plants raised to provide DNA for restriction enzyme digestions (Appendix D.2). At the F$_2$ generation, young leaves were harvested from 150 plants into liquid nitrogen and freeze-dried. This tissue was used for DNA extraction once the colour of seed harvested from F$_3$ plants was identified. Plants were hand-harvested approximately 6 months after seeding, for every generation. One parent of both lines was grown at each generation to provide a seed pigmentation reference check.
I.3.2  Preparation and bulked segregant analysis of genomic DNA

Samples of both parental and progeny DNA were prepared from ca. 80 mg freeze-dried, powdered leaf tissue according to Drost et al. (2001) (extraction buffer, Appendix J.1). The DNA was quantified by fluorometry and diluted to ca. 2 ng µL⁻¹. A bulked DNA preparation to represent the seed color trait was created by selecting ten DNA samples from F₂ progeny that displayed the best seed colour character on the F₃ plants. Equal aliquots were combined in one tube to provide the bulked DNA sample. This process was repeated to create one bulk each of the DNA from yellow- and brown-seeded progeny.

The parental and bulked progeny DNA samples were screened for RAPD polymorphisms by PCR amplification according to Somers et al. (1998). (PCR reaction mixture and thermal cycling program, Appendices J.2 and J.3). A set of 397 random primers (10 nt) was obtained from the University of British Columbia (UBC) for the BSA assays. The PCR products were separated on 2% agarose gels in 1x TAE buffer at 100 V for ca. 3 h, stained with EtBr and photographed under UV illumination. Primers displaying a polymorphism between both the Y and B bulked DNA and the parental DNA were re-tested with the entire progeny set of DNA and parental DNA to determine whether the polymorphism was associated with the yellow- or brown-seeded trait. Polymorphic bands that occurred frequently in only one set of the progeny DNA and the respective parental DNA were evaluated for linkage to the seed colour trait using 'Mapmaker' (ver 3.0b) (Lander et al., 1987).

I.3.3  Evaluation of RAPD markers in B. carinata PGRC/E 21164

Polymorphisms consistent with the seed coat pigmentation trait were found with five of the 397 primers screened (UBC¹¹⁸, UBC¹⁵⁷, UBC³⁵⁰, UBC⁴⁵⁷, and UBC⁵⁴²).
However, according to the Mapmaker program, UBC\textsuperscript{157} (\textsuperscript{5}\text{CGT GGG CAG G}\textsuperscript{3}) produced a fragment only distantly linked to the seed coat colour trait (37.1 cM). The other four primers mapped to one linkage group (greatest distance, 10.7 cM), associated with an undetermined trait. Overall, there was very little DNA polymorphism between DNA samples from the yellow- and brown-seeded progeny. In addition, as discussed in Chapter 5 (section 5.4), inclusion of DNA from heterozygous plants producing yellow seed in the bulked DNA samples likely masked seed coat-related polymorphisms. For these reasons, (the low level of polymorphism and the inclusion of heterozygotes in the bulked DNA) the discovery of a molecular marker linked to the seed pigmentation trait was not successful.
Appendix J  Reagent preparation for RAPDs

J.1  Extraction buffer for micro-DNA preparations

1 M Tris (pH 8.0)  5.0 mL
3 M KCl  16.5 mL
0.5 M EDTA (pH 8.0)  1.0 mL
Water  26.25 mL

J.2  Master mix PCR reagents

Amounts shown are for one reaction

Taq DNA polymerase (5U µL⁻¹)  0.2 µL
10x Tris buffer (pH 8.0)  2.0 µL
25 mM MgCl₂  2.0 µL (2.5 mM)
dNTPs §  2.0 µL (0.2 mM each)
DNA [2 ng µL⁻¹]  4.0 µL
(Alternatively primer  4 µL) †
Sterile water  5.8 µL

§ dNTPs. deoxy-nucleic acids. (as ATP, GTP, CTP, TTP); combined into one 100 mM stock solution; † primer is added to the master mix instead of DNA when the constituents of the bulks or the progeny DNA samples are tested.

J.3  Amplification of genomic DNA for RAPDs (with random primers)

<table>
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<tr>
<th>Step</th>
<th>Temp (°C)</th>
<th>Time (min)</th>
</tr>
</thead>
<tbody>
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</tr>
<tr>
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<td>95</td>
<td>0:20</td>
</tr>
<tr>
<td>3</td>
<td>36</td>
<td>1:00</td>
</tr>
<tr>
<td>4</td>
<td>Ramp 1°C sec-1 to 72°C</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>72</td>
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</tr>
<tr>
<td>7</td>
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</table>

200
Appendix K  Polymorphism as a measure of genetic diversity

K.1 Introduction

Molecular techniques can also be used to measure diversity within and among species. This can be of value to plant breeders using DNA polymorphism to develop a molecular marker for a specific phenotype, such as yellow-seeded *B. napus* or to plant taxonomists for classifying closely-related subspecies. Without genomic diversity, detection of DNA polymorphisms related to a desired trait can be difficult.

Genetic relatedness was determined with random genomic clones from RFLP hybridization patterns that showed a high degree of polymorphism within each species of closely-related accessions of cultivated forms of *B. napus*, *B. rapa* and *B. oleracea* (Figdore *et al.*, 1988). More recently, Diers *et al.* (1994) confirmed distinctive RFLPs in the Brassicaceae when 83 *B. napus* accessions were screened, using 43 genomic DNA clones. Each accession was uniquely identified by RFLP markers, except in near-isogenic cultivars (*e.g.*, Triton and Tower).

K.2 Genetic diversity in *B. carinata*

Currently, many plant genetic resources are being evaluated for genomic polymorphisms using several techniques (RAPDs, RFLPs, amplified fragment length polymorphism [AFLPs]). However, a study on the genetic diversity of *B. carinata* has not been reported by using either RFLP or RAPD technology. Results from the search for a molecular marker related to seed colour in *B. carinata* PGRC/E 21164 demonstrated that there was very little DNA polymorphism in this accession. In order to assess the amount of polymorphism among populations of phenotypically diverse *B. carinata*, six other PGRC/E accessions were obtained from Dr. K. Falk, AAFC. Under field conditions, traits such as seedling vigour, days to flowering, pod filling, plant
height and leaf morphology were variable (Falk, 1999). Three accessions were yellow-seeded, (cv Dodolla, 200417, 21156) and three were dark brown-, (200423, 21276), or light brown-seeded, (cv S-67). The *B. carinata* Y and B lines were included in this assessment of genetic polymorphism. Ten plants from each of these accessions were grown to a four leaf stage in the greenhouse and harvested as before (Appendix I.3). For every accession, the DNA extraction was made from a composite sample of equal weight (ca. 50 mg) of freeze-dried leaves from all 10 plants. The frequency of polymorphic DNA sequences in the eight samples of *B. carinata* was assessed, following the same methods for DNA preparation and evaluation as described earlier for the *B. carinata* progeny (Appendix I.3.2), with the exception that 40 primers rather than 397 were assessed.

Overall, these accessions were not polymorphic (Figure K.1, A). Eleven primers displayed one polymorphism and one primer (UBC303), displayed five polymorphic fragments (Figure K.1, B). There were no apparent subgroup relationships evident within the *B. carinata* accessions, such as might be related to seed colour or plant height. Despite the phenotypic variability, the accessions examined in this study could represent closely-related ancestral germplasm from the Ethiopian Plant Gene Resource Centre.
Figure K.1. Random amplification of genomic DNA from eight *Brassica carinata* accessions.

Accessions grouped in order (lanes 2-9; 10-17; 18-25) as follows:

- PGRC/E 21156, PGRC/E 200417, cv Dodola, Y line 21164.
- B line 21164, cv S-67, PGRC/E 21276, PGRC/E 200423

Ladder (lane 1), 'Low Mass DNA' (Gibco-BRL).

(A) Low diversity PCR amplifications with primers UBC\(^{82}\), UBC\(^{89}\), UBC\(^{147}\)

(B) Polymorphic bands (↕) in PCR amplifications with primers UBC\(^{241}\), UBC\(^{303}\)