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Pigmentation in the developing seed coat and seedling leaves of *Brassica carinata* is controlled at the dihydroflavonol reductase locus

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Abstract

Flavonoid differences between near-isogenic lines of yellow- and brown-seeded *Brassica carinata* were used to identify a genetic block in seed coat and seedling leaf pigment biosynthesis. Seed coat pigment in the brown-seeded line consisted of proanthocyanidins (condensed tannins), while anthocyanin was absent. Dihydroquercetin, dihydrokaempferol, quercetin and kaempferol accumulated only in the mature seed coat of the yellow-seeded line, indicating dihydroflavonol reductase (*DFR*) as an element of genetic control in pigment biosynthesis. *DFR* transcripts from the developing seed coat in the yellow-seeded line were absent or less abundant at 5–30 days after pollination compared to transcript levels in the brown-seeded line. Seedling leaves of the yellow-seeded line exhibited reduced expression of *DFR* and contained less anthocyanin compared to the respective tissues from plants of the brown-seeded line when grown at 25/20 °C (day/night). Cooler (18/15 °C) growing temperatures affected seedling leaf pigmentation, mature seed coat colouration and *DFR* expression in the yellow-seeded line. Comparable brown-seeded line tissues were unaffected by these temperature changes. These results are suggestive of a temperature-sensitive regulator of *DFR* in the yellow-seeded line of *Brassica carinata* which ultimately affects the formation of pigments in the seedling leaves and in the mature seed coats.

Keywords: *Brassica carinata*; A. Braun.; Brassicaceae; Ethiopian mustard; Flavonoid; Proanthocyanidin; Condensed tannin; Dihydroflavonol reductase gene expression

1. Introduction

The success of Canadian-grown canola (predominantly, *Brassica napus* L.) is related to the desirable qualities of the canola oil and its international marketability; however, canola requires improvements in both yield and meal quality characteristics in order to stay competitive (Downey and Rakow, 1987; Slominski, 1997; Rakow et al., 1999). An important strategy to achieve such improvement depends upon the development of yellow-seeded cultivars: cultivars with an unpigmented seed coat. Seed meal analyses have shown that oil concentration increases and fibre and antinutritional polyphenolics (e.g. proanthocyanidins [PA], also known as condensed tannins) decrease in yellow-seeded cultivars of *Brassica rapa* L., *B. juncea* (L.) Czern., and *B. carinata* A. Braun. compared to corresponding dark-seeded cultivars (Shirzadegan and Röbbelen, 1985; Simbaya et al., 1995). However, a low fibre, yellow-seeded (PA-free) *B. napus* has proven difficult to develop due to poorly-defined seed coat pigment composition and the multiple recessive genes that control most unpigmented seed coat phenotypes (Shirzadegan, 1986; Chen et al., 1988; Wang and Liu, 1991).

Brassica carinata, an Ethiopian mustard seed, differs from the other *Brassica* species because the yellow-seeded phenotype is inherited as a monogenic, dominant trait (Getinet and Rakow, 1997). This species provides a naturally-occurring model of pigment repression and is ideal for a study of seed coat pigment synthesis using near-isogenic lines. A yellow-seeded cultivar of *B. napus* may be more readily developed through interspecific crosses with *B. carinata* once the genetic block for pigment synthesis is identified in the latter species. Thus,

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the objective of this research was to determine pigment-related flavonoid composition in the seed coat from a yellow-seeded (unpigmented seed coat) line of *B. carinata* ('Y line') compared to those of a near-isogenic brown-seeded line ('B line'). The differences suggested a control point at the dihydroflavonol reductase (*DFR*) gene. Subsequent comparison of *DFR* transcript levels in developing seeds and seedling leaves of the two lines, as well as anthocyanin analysis in the seedlings, confirmed this gene as a locus of control of pigment biosynthesis in both tissues.

2. Results and discussion

2.1. Flavonoid accumulation in the seed coat of yellow-seeded Brassica carinata

The mature seed coat extract from the Y line contained dihydroquercetin together with small amounts of dihydrokaempferol, *t*-cinnamic acid and two flavonols, quercetin and kaempferol (Fig. 1; Table 1). Trace amounts of dihydromyricetin were discovered by monitoring the extract using single ion recording (SIR) during mass analysis (Table 1). Myricetin was not detected in any samples. The B line seed coat extract did not contain any of these metabolites except for trace amounts of kaempferol detected by SIR (data not shown). The most abundant phenolic compounds (sinapic acid and a methyl ester of sinapic acid) were common to both Y and B line seed coat extracts (Fig. 1), but did not appear in embryo-free extracts made from hand-dissected seed coat (data not shown) (Marles, 2001).

Accumulation of flavonoid intermediates has been reported in unpigmented seed coats of legumes, barley (*Hordeum vulgare* L.) and *Arabidopsis thalina* (L.) Heynh. Flavones, flavonols and dihydroflavonols accumulated in the seed coats of white bean (*P. vulgaris*) and *Vicia faba* L. cv Blandine, whereas only PA was present in the dark-seeded *V. faba* cv Alfred (Bekkara et al., 1998; Beninger and Hosfield, 1998; Beninger et al., 2000). Several *ant* mutant lines of barley with unpigmented, PA-free testa, contained increased levels of flavones compared to the wild type and the *Arabidopsis*

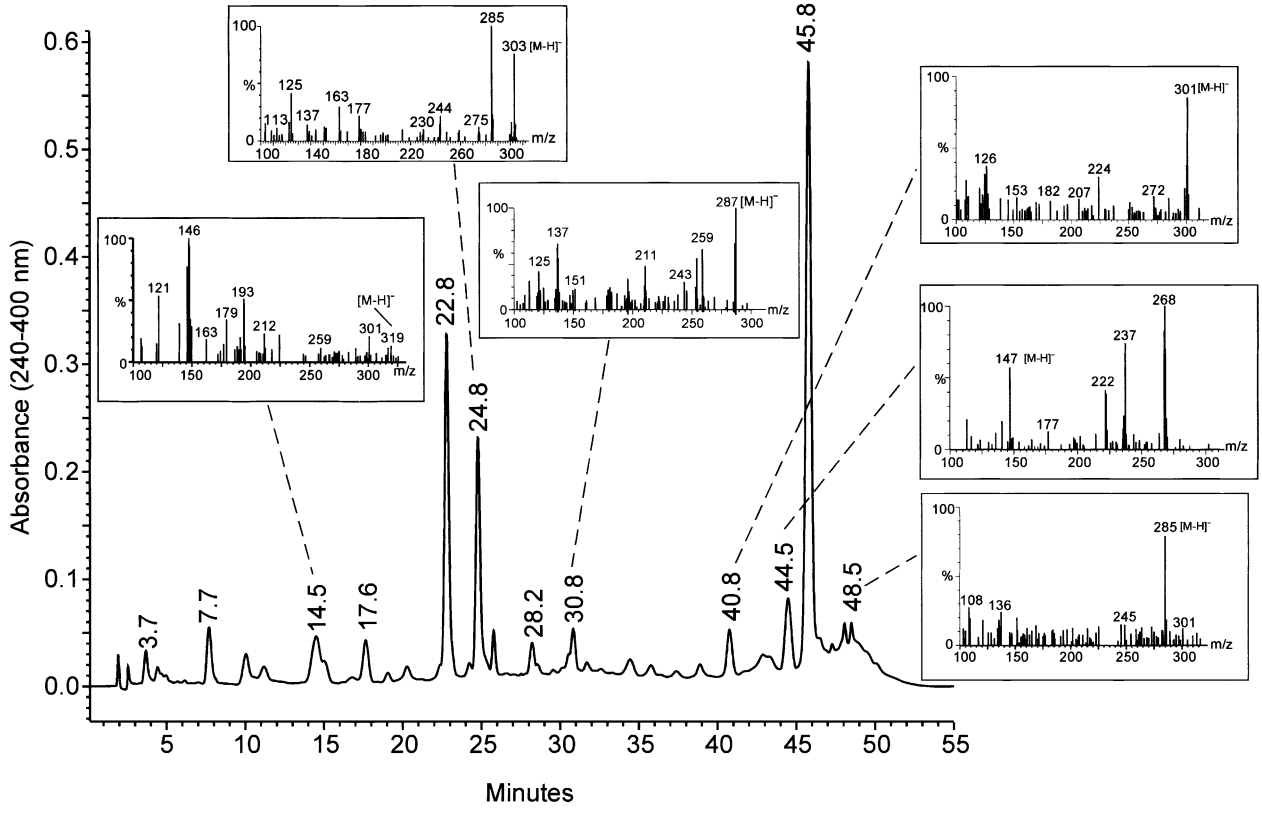

Fig. 1. Resolution of phenylpropanoids and flavonoids in seed coats of yellow-seeded *Brassica carinata* line. RP-HPLC retention time (min) of UV-detectable compounds using an MeCN gradient modified with trifluoroacetic acid; see Section 4 for additional conditions. Metabolites were identified from the ethyl acetate-soluble fraction of a methanol extract of the Y line of PGRC/E 21164 *Brassica carinata*: dihydromyricetin (detectable by SIR only) (14.5 min), dihydrokaempferol (30.8 min), dihydroquercetin (24.8 min); quercitin (40.8 min), *t*-cinnamic acid (44.5 min) and kaempferol (48.5 min) were detected as shown. Derivatives of sinapine, likely associated with residual embryo material attached to the seed coat, were identified at retention times of 22.8 min (sinapic acid) and 45.8 min (methyl ester of sinapic acid). Compounds were identified by comparison with RT and UV-spectra of authentic standards and by LC–MS–MS (mass spectra of peaks shown in boxes). Chromatogram is presented as a plot of maximum absorbance (240–400 nm) and is representative of three or more injections.

Table 1
Compounds identified by LC–MS–MS in extracts of mechanically-collected seed coats of *Brassica carinata* Y and B lines

Compound name	Retention time (min)	Molecular mass (Da)	Mass spectrum (m/z)[a]			
			Parent ions[b]		In-source fragments	Daughter ions[b]
			$[M+H]^+$	$[M-H]^-$		
Y line extract (100% MeOH fraction)						
Dihydromyricetin[c]	13.3	320	321	319	193, 125, 179, 301	192, 93, 181, 301, 151
Sinapic acid	20.0	224	225	223	208, 164, 179	193, 208, 164, 149, 125
Dihydroquercetin	22.6	304	305	303	285, 163, 125, 177, 275	125, 176, 285, 302
Dihydrokaempferol	28.1	288	289	287	259, 125, 151	259, 125, 177, 151, 107
t-Cinnamic acid	39.6	148	131	147	103	146, 103, 77, 59
Sinapic acid ME[d]	40.4	239	239	237	222, 207	207, 208, 222, 179
Quercetin	40.5	302	303	301	151, 179	151, 179, 121, 107, 93, 273
Kaempferol	48.5	286	287	285	–	93, 136, 151, 161, 257, 108
B line extract (total extract)						
Sinapic acid	19.7	224	225	223	208, 164, 179	193, 149, 163, 207, 125
Sinapic acid ME	39.7	239	239	237	222, 207	207, 222, 179
Kaempferol[e]	44.9	286	287	285	–	93, 136, 161, 108, 256, 151

[a] The most abundant fragments (>25% relative abundance), arranged in order of percent relative abundance; in-source fragmentation shown for $[M-H]^-$ only.
[b] Fragmentation patterns relate to the parent molecule ion $[M-H]^-$; $[M+H]^+$ parent ions do not fragment well and these daughter ions are not shown.
[c] Dihydromyricetin was present in trace amounts only and was detected by SIR.
[d] ME, methyl ester, an artifact of the extraction (e.g. a hydroysis product of sinapine).
[e] Determined by SIR in the B line using the 50% MeCN gradient method.

transparent testa mutants were shown to accumulate higher levels of quercetin and kaempferol than the wild type plants (Shirley et al., 1995; Pelletier et al., 1999; Jende-Strid, 1993). The accumulation of flavonols may be prevalent among *Brassica* cultivars with unpigmented seed coat, since analyses of yellow-seeded *B. juncea* seed coat aglycones similarly showed that flavonols were abundant (Marles, 2001).

2.2. Differential pigment accumulation in seedlings and the mature seed coat

Young seedlings from the Y line of *B. carinata*, grown at warm temperatures had lighter green leaves at the two- to four-leaf stage compared to the darker bluish-green leaves of the near-isogenic B line seedlings (Table 2; Fig. 2A and B). This difference in pigmentation applied only to true leaves and was correlated with a difference in the anthocyanin content. A pelargonidin-like anthocyanin and a second, unidentified; anthocyanin were produced in B line seedling leaves and were absent in the Y line extracts under these conditions (Table 3; Fig. 2C and D). After the five-leaf stage, seedling leaf colour in the Y line was comparable to that of the leaves of the B line (data not shown).

Seed that matured under consistently warm temperatures was tan-yellow, whereas mature seed from the B line was deep brown (Fig. 2E and F). The pigments in the mature seed coat of the B line were unextractable in sufficient quantities to allow detailed identification of polymer structure. However, acidic hydrolysis using *in situ* histochemicals showed that PA was present in the B line seed coat, but was absent in the Y-line (Fig. 2G and H). Anthocyanins in the seed coat were absent in both lines. In situ histochemistry was chosen to determine the phytochemical nature of the unextractable pigment in mature *B. carinata* seed coat, since this method was successfully used to identify PA in the seed coat of several legume species and barley (Aastrup, 1985; Lees et al., 1993; Skadhauge et al., 1997). A histochemical survey of the seed coat pigments and patterns also differentiated

Table 2
Colour of the leaf tissue and mature seed of *Brassica carinata* grown under two temperature regimes

Sample	Tissue colour	Colour reference number[a]
Warm conditions (25/20 °C)[b]		
Y line seedling leaf	Light green	27B7
B line seedling leaf	Bluish green	26D5
Y line seed	Tan yellow	4C8
B line seed	Deep Brown	7F8
Cool conditions (18/15 °C)[b]		
Y line seedling leaf	Bluish green	26D5
B line seedling leaf	Bluish green	26D5
Y line seed	Light brown	5C8
B line seed	Deep brown	7F8

[a] Kornerup and Wanscher (1963); see Section 4.1.2.
[b] Day/night temperatures.

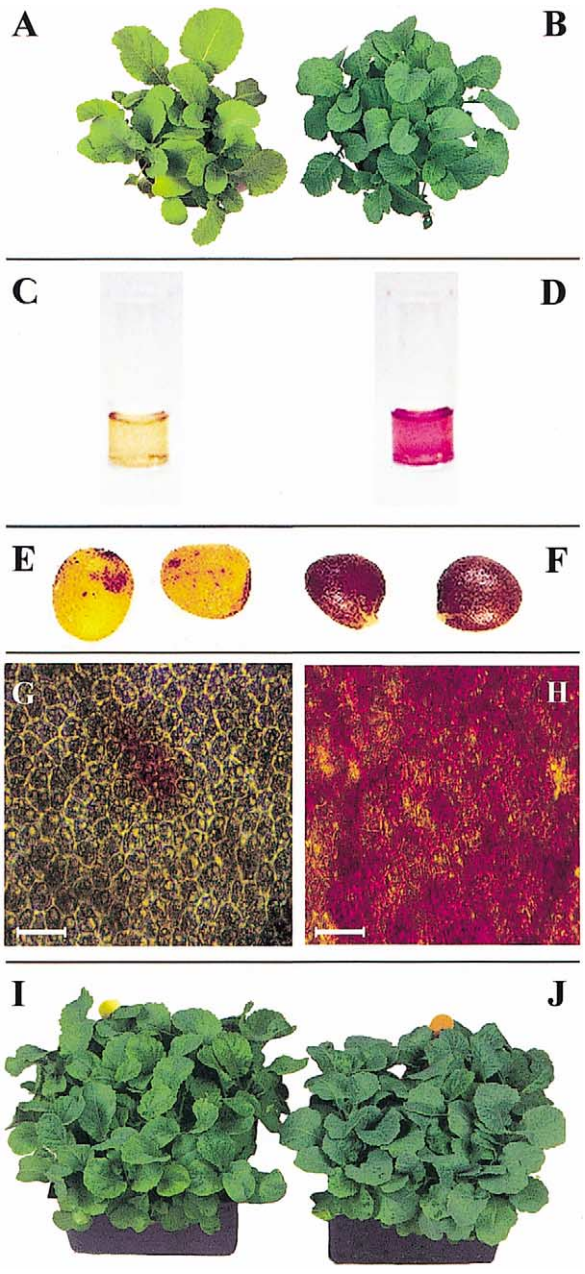

Fig. 2. Pigment accumulation in *Brassica carinata* seedling leaves and the mature seed. (A) Light green leaves at the four-leaf stage of *Brassicca carinata* PGRC/E 21164 Y line from warm-growing (25/20 °C) conditions. (B) Leaves of near-isogenic B line at the four-leaf stage were a darker bluish-green under the same conditions. (C) Isoamyl alcohol extract from Y line leaf tissue contained no detectable anthocyanins. (D) Anthocyanins were visible in the isoamyl alcohol extracts of the B line (confirmed by spectral analyses and co-migration of authentic standards on TLC plates, Table 3). (E) Freshly imbibed seed from *B. carinata* Y line, largely unpigmented. (F) Dark red–brown colour of freshly imbibed seed from the near-isogenic B line. (G) Histochemical staining of the seed coat dissected from Y line indicated that both anthocyanins and proanthocyanidins were absent; bar, 100 μm. (H) Histochemical staining of the seed coat dissected from the near-isogenic B line indicated that red proanthocyanidins were present and anthocyanins were absent; bar, 100 μ. (I) Darker green leaves of Y line under cool-growing (18/15 °C) conditions are nearly identical to leaves of B line (**J**) at the four-leaf stage, grown at the same time.

between PA and anthocyanin pigmentation from a selection of other Brassicaceae (Marles, 2001).

The unextractable nature of PA is well-known in the seed tissues of other plant species. Pigment was reported to be unextractable in maize (*Zea mays* L.) pericarp and floral organs, in the seed coat of dark red kidney bean (*Phaseolus vulgaris* L. cv Montcalm) and in black-seeded *B. napus* cv Tower (Leung et al., 1979; Styles and Ceska, 1989; Grotewold et al., 1994; Beninger and Hosfield, 1999). The normally colourless PA undergoes secondary changes during seed maturation to form insoluble complexes with the cell wall and other phenolics in the seed coat, causing darkening (Beninger and Hosfield, 1999). This darkening process was also proposed for PA in the brown testa of the caryopses of sorghum (Stafford, 1990).

Differential pigment accumulation was temperature-dependent in yellow-seeded *B. carinata*. When Y line seedlings were grown at 18/15 °C or were chilled overnight, the new leaves produced a pelargonidin-like anthocyanin and were similar in colour to the leaves of B line seedlings (Table 2, Fig. 2I and J). However, the Y line leaves did not produce detectable amounts of the second (unidentified) anthocyanin found in the B line leaf extracts.

The mature seed from the Y line was light brown rather than yellow when plants flowered and set seed under cooler temperatures. This demonstrated that temperature influenced seed coat pigmentation in yellow-seeded germplasm. Similar temperature-related effects on seed coat pigment biosynthesis occurred in a doubled-haploid, brown-seeded population of *B. napus* in which mature seeds were light brown from plants grown at 24/20 °C and dark brown from plants grown at 16/12 °C (Van Deynze et al., 1993). However, seed colour in our brown-seeded lines of *B. carinata* and in the black-seeded populations of *B. napus* (Van Deynze et al., 1993) was not noticeably affected by the growing temperature.

2.3. *DFR* gene expression in the seedling leaves and the developing seed of *B. carinata*

The accumulation of dihydroquercetin and small amounts of other flavonoids in seed coat of yellow-seeded *B. carinata* suggested *DFR* as a locus of control for pigmentation in this species. Flavonoid transcript abundance was too low in developing *B. carinata* seed to detect a signal by Northern hybridization. Thus, quantitative reverse transcription-PCR (RT-PCR) assays were used to determine whether *DFR* gene expression correlated with the unpigmented seed phenotype observed in *B. carinata* under warm growing conditions. *DFR* transcripts appeared to be reduced in all stages of seed development (flower bud [fb] to 30 days after pollination [dap]) in the Y line compared to the same tissue stage in the B line (Fig. 3A). The maximal accumulation of *DFR* transcript occurred later in the developing seed of the Y line (around 20 dap), whereas

Table 3
One-dimensional TLC analysis of MeOH–HCl extracts from seedling leaves of *Brassica carinata* grown in two temperatures regimes

Plant line/temperature (day/night, °C)	Extract colour (unpurified)	R_f		Absorbance maxima[a]
		BAW[b]	Forestal[c]	
Y line				
25/20	No detectable colour			
18/15	Pink–orange	94	77	280, 430[sh], 556
B-line				
25/20	Pink–orange	60, 90[d]	77	280, 430[sh], 550
18/15	Pink–orange	60, 94[d]	77	280, 430[sh], 556
Standards				
Pelargonidin	Orange–red	95	76	280, 435[sh], 556
Malvidin	Red–purple	78	75	270, 430[sh], 550
Delphinidin	Blue–purple	63	36	270, 428[sh], 535
Peonidin	Red–purple	40	74	270, 430[sh], 540
Cyanidin	Rose–red	84	57	274, 435[sh], 550

[a] Spectral data of unpurified extract and standards recorded from 190 to 700 nm in MeOH–HCl prior to TLC separation.
[b] BAW, *n*-butanol–acetic acid–water (4:1:5).
[c] Forestal, conc. HCl–acetic acid–water (3:30:10).
[d] Two compounds resolved in BAW separation of the B line. Subsequent 2-D separation (first, 15% HCl; second, Forestal) of the B line extract revealed one compound co-migrated with pelargonidin. The second compound did not co-migrate with any of the standards.
[sh] Shoulder.

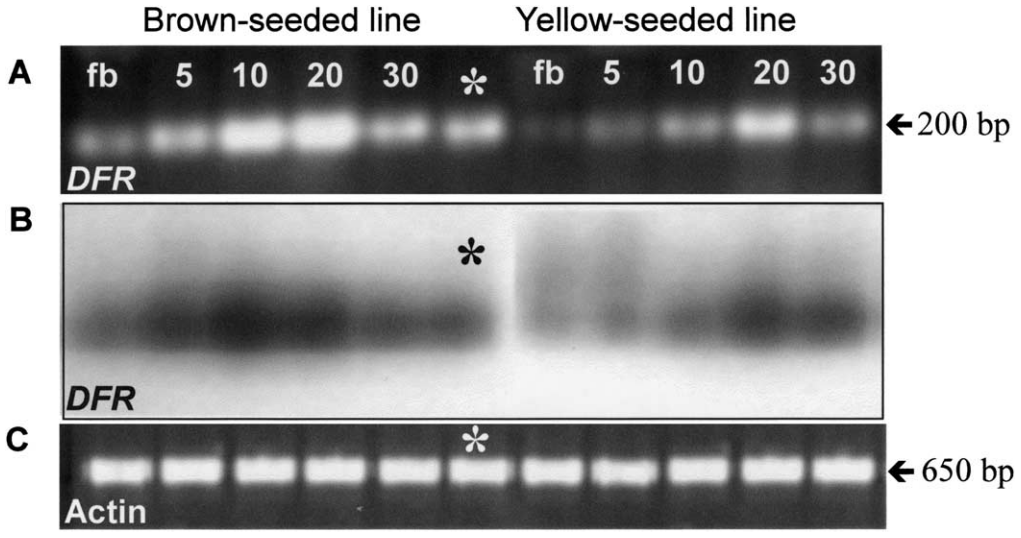

Fig. 3. Expression of *DFR* in the developing seeds from the near-isogenic Y and B lines of *Brassica carinata*. (A) *DFR* fragment (200 bp) amplified by RT-PCR from *DFR* transcripts in developing seed (ethidium-stained gel). (B) Southern blot of the RT-PCR gel hybridised with a radioactive probe derived from *B. carinata* PGRC/E 21164. (C) RT-PCR quantification: actin quantification fragment (650 bp) amplified from same RT reaction as *DFR*. Lanes marked *, RT-PCR-amplified *DFR* cDNA using mRNA from seedling leaf tissue (positive control). Lanes marked 'fb to 30' represents amplification products from flower bud (fb) and seed harvested 5–30 days after pollination (dap) from warm-grown plants.

DFR expression peaked around 10 dap in the B line. Transcript level appeared to be maintained in the B line until at least 20 dap, decreasing by 30 dap. Hybridization of a radioactive *DFR* probe to the RT-PCR fragment (200 bp) confirmed that this amplicon was homologous to *DFR* (Fig. 3B). Sequence analysis of this amplified *DFR* fragment (NCBI AF464874) indicated 96% similarity to *DFR* from *B. oleracea* (NCBI AF229385) and 89% similarity to *Arabidopsis DFR* (NCBI AJ 251982). At this point, we do not fully understand the mechanism of repression of proanthocyanidins in yellow-seeded germplasm and its relationship to reduced *DFR* transcript level. Repression of *DFR* was reported in the *Arabidopsis tt3* mutant, in which the *DFR* gene was altered, and in the *tt8* mutant, in which a basic helix-loop-helix regulatory gene disrupted expression of *DFR* and transcript levels were reduced (Shirley et al., 1992; Nesi et al., 2002).

Accumulation of *DFR* transcript was also examined in the Y line seedlings grown under warm conditions. Expression was reduced from the cotyledon stage to the

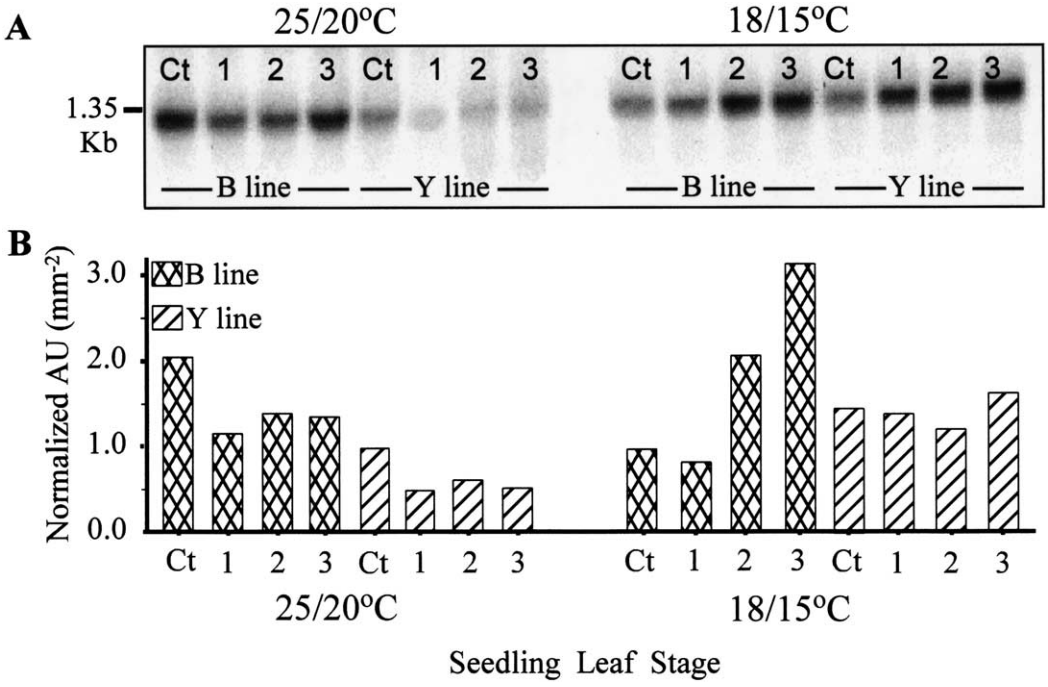

Fig. 4. Expression of *DFR* in the seedling-leaf tissue of *Brassica carinata*. (A) Representative Northern blot of *B. carinata* total RNA extracted from the cotyledon (Ct) and leaf tissue (first leaf fully emerged, 1, to third leaf fully emerged, 3) probed with *DFR*. Blot probed with cloned *B. carinata* *DFR* fragment and exposed to film for 48 h. (B) For quantification of *DFR* expression, the intensity (absorbance/mm^{-2} [AU mm^{-2}]), of the *DFR* signal on the blot was normalized to the respective actin signal and plotted.

third-leaf stage compared with similar tissues of the B line (Fig. 4A). Reduced gene expression in the Y line corresponded with the absence of anthocyanins in these tissues (Fig. 2). Subsequently, the level of *DFR* transcripts was measured at all leaf stages in cool-grown seedlings to determine whether gene expression was affected by temperature. Under the cooler conditions, DFR transcript level in the Y line seedlings was consistently higher compared to the warm conditions and correlated with the presence of anthocyanins (Fig. 4A).

DFR transcript level remained constant in the Y line from the cotyledon stage to the third leaf stage, although it increased in cool-grown seedlings of the B line. Although the leaf colour appeared visually similar between both lines under the cool conditions, the B line

Table 4
Molecular ions and fragmentation patterns of daughter ions of authentic standards analyzed by Quattro LC–MS–MS system[a]

Compound name	Retention time[b] (min)	Molecular mass (Da)	Mass spectrum (m/z)[c]			
			Parent ions		In-source fragments	Daughter ions[d]
			$[M+H]^+$	$[M-H]^-$		
Sinapine	3.7	295	296	294	223, 111, 179, 208, 150, 125	223, 237, 150, 208, 179, 111
Dihydromyricetin	13.2	320	321	319	193, 179, 301, 151, 125, 257	124, 151, 181, 179, 193, 301
Sinapic acid	20.0	224	225	223	208, 164, 179	193, 208, 164, 149, 125
Dihydroquercetin	22.5	304	305	303	285, 125, 177, 154, 259, 275	83, 125, 151, 175, 285, 302
Dihydrokaempferol	28.9	288	289	287	259, 125, 269, 243	259, 125, 177, 151, 107
t-Cinnamic acid	39.4	148	131	147	103, 77	103, 145, 77
Quercetin	40.4	302	303	301	151, 179	151, 179, 121, 107, 93, 273
Kaempferol	48.2	286	287	285	–	93, 136, 151, 161, 257, 108
Kaempferol[e]	44.9	286	287	285	–	93, 136, 161, 108, 256, 151

[a] Standards obtained from Apin Co., London, UK.
[b] Retention time (min) according to PDA-detection on LC–MS MeCN gradient modified with formic acid.
[c] The most abundant fragments (>25% relative abundance), arranged in order of percent relative abundance; in-source fragmentation shown for $[M-H]^-$ only.
[d] Fragmentation patterns relate to the parent molecular ion $[M-H]^-$; $[M+H]^+$ daughter ions are not shown.
[e] Determined using 50% MeCN gradient method.

produced more than twice as much *DFR* transcript compared to the Y line at the third leaf stage (Fig. 4B). These temperature–related effects on *DFR* expression in *B. carinata* were unexpected and have not been reported for seed colour mutants of other species.

3. Conclusions

The genetics and enzymology of phenylpropanoid and flavonoid biosynthesis have been studied intensively using flower and seed colour mutants of petunia, snapdragon (*Antirrhinum majus* L.), maize, barley, *Arabidopsis* and other genera, and a highly regulated, biochemically complex pathway is emerging (Holton and Cornish, 1995; Mol et al., 1996). The yellow-seeded and brown-seeded near-isogenic lines of *B. carinata* used in this study have provided new tools with which to examine the regulation of flavonoids in *Brassica* species. Our gene expression results indicate that down-regulation of *DFR* occurs in both seedling and developing seed tissue of yellow-seeded *B. carinata* at warm temperatures. Together with the flavonoid composition data, seed coat pigmentation data, and the sensitivity to temperature, they also suggest that *DFR* in *B. carinata* is controlled by a temperature-sensitive regulator. Various authors have suggested that several proteins must be co-ordinately expressed for tissue pigmentation to occur (Kubasek et al., 1992; Grotewold et al., 1994). A regulatory cascade which includes both temperature-induced and developmentally-induced proteins would explain the pigmentation patterns in young leaves and developing seed coats of yellow-seeded *B. carinata*.

4. Experimental

4.1. Plant materials

4.1.1. Development of near-isogenic lines of Brassica carinata A. Braun.

The accession PGRC/E 21164 was provided by Dr. Gerhard Rakow, Agriculture and Agri-Food Canada, Saskatoon, Canada (AAFC) from bulked seed that originated from germplasm provided to AAFC by Dr. A. Getinet, Plant Genetic Resource Centre, Ethiopia (PGRC/E). *B. carinata* PGRC/E 21164 seed segregating for yellow- and brown-seeded phenotypes was planted in a soiless mix (Redi-Earth, Grace & Co., Ajax, ON) and placed in a controlled-environment growth-chamber (Conviron, Winnipeg, Canada) under fluorescent and incandescent lighting (320 to 510 μE, Licor Model LI-185B), with an 18 h photoperiod (22 °C/20 °C light/dark). Subsequent generations (S_2, S_3) were developed from pure breeding yellow- and brown-seeded lines, each grown from one yellow- and one brown-seeded S_1 plant and propagated in the growth-chamber as described for the first generation. A yellow-seeded line (the 'Y' line) and a brown-seeded line (the 'B' line) were used for chemical and molecular analyses.

4.1.2. Colour classification of seed and leaves

Seed and leaf colour was classified using a numbered rating system by comparing the plant tissue to colour plates, according to the directions in the Methuen Handbook of Colour (Kornerup and Wanscher, 1963). The colour number specifically refers to the colour plate number, column letter and row number.

4.1.3. Developing seed for monitoring gene transcripts

Tissue was harvested from *B. carinata* PGRC/E 21164 Y line and B line plants grown in a soiless mix in a greenhouse under fluorescent light banks (VHO bulbs [Phillips™], supplemented with 100 watt incandescent light [GE™]; 200–300 μE) with an 18 h photoperiod (25 °C/20 °C light/dark). Controlled pollination was performed on five or six racemes per plant, then all subsequent floral meristems were removed. Developing seed was dissected from the siliques (including unopened buds suitable for pollination) at 5, 10, 20 and 30 dap and immediately frozen in liquid nitrogen for storage at −80 °C.

4.1.4. Seedling leaf tissue for anthocyanin determination and RNA extraction

Seedlings of *B. carinata*, Y and B lines were grown to the fourth-leaf stage in the greenhouse as described for developing seed. A second set of plants were grown under similar illumination but at cooler temperatures (18 °C/15 °C light/dark). Leaves were harvested at the 2–3 leaf stage, ca. 12 days after germination and frozen as 2-g samples for anthocyanin analyses. In addition, 200-mg leaf samples from the plants described above were harvested and immediately frozen in liquid nitrogen for RNA extractions. Leaves were collected at four different stages starting ca. 8 days after germination: 'Ct', cotyledons fully developed; '1', first leaf emerged and opened; '2', second leaf to open, third leaf beginning to emerge; '3' third leaf opened; fourth leaf visible.

4.2. Anthocyanin extraction and analysis

Frozen leaves and whole seeds (2 g) were ground in liquid nitrogen and extracted immediately for anthocyanins, using an *iso*amyl alcohol method (Harborne, 1984). Another extraction with 2 g tissue was made using 1% HCl in MeOH (MeOH–HCl), heating in a waterbath (90 °C) for 10 min followed by partioning (4×) against EtOc and reserving the lower phase for concentration by rotary evaporation. Subsequently, a wavelength scan (190–700 nm) was taken of each reconstituted extract (1 ml, 100% MeOH) (Cary Bio 3, Varian,

Rocklin, CA), prior to one-dimensional (1-D) separation on cellulose TLC (BAW, *n*-butanol–acetic acid–water (4:1:5) or Forestal, conc. HCl–acetic acid–water (3:30:10) solvents) (Harborne, 1984). Resulting components from 1-D chromatography were scraped and eluted in 100% MeOH and subjected to two-dimensional (2-D) separation, 15% acetic acid followed by Forestal.

4.3. In situ histochemical detection of anthocyanins and condensed tannins

Seed coat tissue was soaked (4 °C, 1–2 h in distilled water) and hand-dissected. Histochemical stains (BuOH–HCl [70:30, v/v], MeOH–HCl, EtOH–HCl [2:1, v/v] and freshly prepared 1% vanillin in 6 N HCl) were differentially applied to distinguish PA from anthocyanins according to the methods of Lees et al. (1993), Skadhauge et al. (1997) and Aastrup (1985). Observations were collected from 10 replications for each reagent.

4.4. Extraction and fractionation of flavonoids and phenylpropanoids from B. carinata

Seed coat tissue used for metabolic profile analyses by HPLC and LC–MS–MS was mechanically collected using the tangential abrasive dehulling device (Oomah et al., 1981). Mechanically-collected seed coat samples (78 g, Y line and 76 g, B line, *B. carinata*) for HPLC analyses were extracted in 80% aqueous MeOH and hydrolyzed in 2 N HCl according to standard methods for isolating aglycones (Mabry et al., 1970; Harborne, 1984). The hydrolyzed extract was partitioned 4× against EtOAc and dried over Na_2SO_4. The Y line was further fractionation by sequentially washing the EtOAc residue with (1) water, (2) 30% aq. MeOH and (3) MeOH. The residual EtOAc-soluble extract remaining after each wash was taken to dryness by rotary evaporation between each wash step. The B line was analyzed as an unfractionated, EtOAc-soluble extract only.

4.5. Analytical RP-HPLC

Samples (10 and 20 μl) were separated using a Waters 2690 'Alliance' liquid chromatograph (Milford, MA) equipped with an autosampler, a 996 PDA detector, and a heated (30 °C) Symmetry reversed-phase (RP) C_{18} column (Waters, 3.0×150 mm; 5 μm particle size). Organic solvents were of HPLC-grade (EM Sciences, Gibbstown, NJ); authentic standards were obtained from Apin Co. (London, UK) and dissolved in 100% MeOH; other chemicals were reagent-grade or better. Instrument separations and data analyses were controlled using Waters Millenium 32 software (ver. 3.2). Extracts (5 and 10 μL aliquots) were eluted with a 33-min aqueous-acetonitrile gradient modified with 0.05% trifluoroacetic acid (v/v) (10% acetonitrile (MeCN), 5 min; 40% MeCN, 10 min; hold 40% for 10 min; return to 10% MeCN, 5 min; hold 10% MeCN, 3 min) at 0.4 ml min^{-1}. For late eluting compounds (e.g. kaempferol), a 55-min elution gradient (10% MeCN, 10 min; 20% MeCN, 15 min; hold 20% for 5 min; 30% MeCN, 15 min; return to 10% MeCN, 5 min; hold 10% MeCN, 5 min) was used. A UV-spectral library was constructed by analyzing authentic standards (Apin Co., UK) under the same separation conditions. Selected standards were separated preceding and following extract injections as a check on the reproducibility of retention times. For UV-spectral identifications, 'max plot' chromatograms were generated (240–400 nm).

4.6. Liquid chromatography–tandem mass spectrometry (LC–MS–MS)

Mass analysis was conducted using a 'Quattro LC' with a Z-spray interface (MicroMass Co., Manchester, UK), MassLynx software (ver. 3.4) and an Alliance RP-HPLC system configured as described previously, substituting a water-acetonitrile solvent system modified with 0.1% formic acid (0.2 ml min^{-1}). Sample ionization was achieved using atmospheric pressure chemically-induced ionization (APcI), with instrument settings tuned for preselected parent molecular ion and daughter ion analyses in both negative- and positive-ion mode. Compound identification was confirmed with authentic standards (Table 4).

4.7. RNA extraction

Total RNA from samples of seedling leaf tissue (cotyledons, first to fourth leaf stage), unopened floral buds (fb) and developing seed from 5 and 10 dap was isolated using a Plant RNeasy kit (Qiagen), following the manufacturer's directions. Ground tissue from 20- and 30-dap was extracted using a modified hot phenol method to counteract high viscosity due to seed storage proteins or polysaccharides in the final preparation (Verwoerd et al., 1989; Wilen et al., 1990). Molecular biology-grade chemicals (Sigma, St. Louis, MO.) were used unless otherwise mentioned. The molecular marker ladder for agarose gels was '1 Kb-Plus' (Gibco-BRL, Gaithursburg, MD). Radioisotope (α-^{32}P) was obtained from Amersham (specific activity, 3000 Ci/mmol dCTP).

4.8. Analysis of DFR expression in developing seed by RT–PCR

Aliquots of total RNA (ca. 3 μg) from the fb, developing seed and seedling leaf tissue (positive control) were reverse transcribed using Superscript (Gibco-BRL), following the manufacturer's directions. The protocol was modified by including the 3′ primer for actin (5′ CCC TGC CAT GTA TGT TGC 3′ [$T_m = 67$ °C]; GenBank

Accession No. AF111812, 1998) in the same first-strand synthesis reaction as the 3′ primer for *DFR*. In this way, the generation of *DFR* cDNA was quantified by synthesis of a constitutive transcript from the same set of RNA samples according to established protocols (Ride et al., 1999; Shimizu et al., 1999; Yu et al., 1999).

For first strand synthesis of *DFR*, the 3′ primer was designed from the first exon of *DFR* from *A. thaliana* (5′ CTT CGG GTT TCA TCG GTT CAT 3′ [$T_m = 51$ °C]) (NCBI AB007647). One fragment (200 bp) was amplified in each PCR assay using the same 3′ primer from *A. thaliana* and a 5′ primer (5′ TCC GTT TAT AGC GTC ATC GTA GC 3′, [$T_m = 55$ °C]) from a *B. carinata* genomic sequence (AY093619). The fragment was cloned from preliminary PCR experiments, sequenced and found to be 95% similar to published *DFR* sequences for *B. napus* (AF229383) and 93% for *B. oleracea* [AF229385]). Sequence comparisons were made using the NCBI 'Blastn' database program with the Fasta format (Pearson and Lipman, 1988; Altschul et al., 1990).

PCR amplification (programmable thermal-cycler, model PTC-200, MJ Research Inc., Watertown, MA) was used to detect differences in the pattern of *DFR* transcripts in the RNA samples from developing *B. carinata* seed. The number of program cycles required for logarithmic PCR-amplification of the *DFR* transcript (38) was determined by increasing the number of cycles by two between 28 and 40 cycles. Optimal primer concentrations (0.4 and 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. Amplified products were separated on 2% agarose gels in Tris-acetic acid buffer (Sambrook et al., 1989), stained in ethidium bromide (EtBr) and photographed using a Stratagene gel documentation system (Eagle Eye II; 'Eaglesight' software [ver. 3.2]) under UV illumination (λ_{366}).

4.9. Northern blotting for determination of DFR expression in seedling leaves

Denaturing formaldehyde gels (1.2% agarose) and Northern hybridization followed the protocols outlined by Sambrook et al. (1989). Nucleic acids were transferred by capillary blotting in 10x MOPS to Hybond N^+ membranes (0.45 μm, Amersham Pharmacia, Piscataway, NJ) and hybridized at 40 °C (Hybaid oven, InterScience, Markham, ON) using Ultrahybe (Ambion, Austin, TX), following the manufacturer's directions. For interpretation of gene expression, the density of the test probe (*DFR*) in each lane was normalized to its corresponding control probe (actin) according to Suzuki et al. (2000) (Bio-Rad Calibrated Imaging Densitometer model 710-GS controlled by 'Quantity One' software, ver. 4.1). Northern blots were repeated three times, once for each new set of RNA extracts.

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