Proanthocyanidin biosynthesis in the seed coat of yellow-seeded, canola quality Brassica napus YN01-429 is constrained at the committed step catalyzed by dihydroflavonol 4-reductase

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Proanthocyanidin biosynthesis in the seed coat of yellow-seeded, canola quality *Brassica napus* YN01-429 is constrained at the committed step catalyzed by dihydroflavonol 4-reductase[1]

Leonid Akhov, Paula Ashe, Yifang Tan, Raju Datla, and Gopalan Selvaraj

Abstract: The yellow seed characteristic in *Brassica napus* L. is desirable because of its association with higher oil content and better quality of oil-extracted meal. YN01-429 is a yellow-seeded canola-quality germplasm developed in Canada arising from several years of research. Seed-coat pigmentation is due to oxidized proanthocyanidins (PA; condensed tannins) derived from phenylpropanoids and malonyl CoA. We found PA accumulation to be most robust in young seed coats (20 d post anthesis; dpa) of a related black-seeded line N89-53 and only very little PA in YN01-429, which also contained much less extractable phenolics. The flavonol content, however, did not show as great a difference between these two lines. Furthermore, sinapine, a product of the general phenylpropanoid metabolism, was present at comparable levels in the embryos of both lines. Dihydroflavonol reductase (DFR) activity that commits phenolics to PA synthesis was lower in YN01-429 seed coats. The results of Southern blot and in silico analyses were indicative of two copies of the *DFR* gene in *B. napus*. Both copies were functional in YN01-429, ruling out homeoallelic repression or silencing, but together they showed very low expression levels (17-fold fewer transcripts) relative to *DFR* activity in N89-53 seed coats. These results collectively suggest that YN01-429 differs in regulatory circuits that impact the PA synthesis branch much more than the flavonol synthesis branch in the seed coats and such circuits do not impinge upon general phenylpropanoid metabolism in the embryos.

Key words: condensed tannin, dihydroflavonol reductase, flavonoids, oilseed rape, rapeseed, transparent testa.

Résumé : Le jaune caractéristique des grains du *Brassica napus* L. est désirable, compte tenu de son association avec une haute teneur en huile et une meilleure qualité de la farine extraite de l'huile. Le YN01-429 constitue un germplasme à graine jaune de qualité canola résultant de plusieurs années de recherches. La pigmentation des téguments est attribuée à des proanthocyanidines oxydées (PA; tannins condensés) dérivées de phénylpropanoïdes et du CoA du malonyl. Les auteurs ont trouvé une plus forte accumulation de PA chez les jeunes téguments (20 jours après l'anthèse; dpa) d'une lignée apparentée à grains noirs N89-53 et très peu de PA chez la lignée YN01-429 qui contient à la fois beaucoup moins de phénols extractibles. Cependant, la teneur en flavonols ne montre pas autant de différences entre ces deux lignées. De plus, la sinapine, un produit du métabolisme général des phénylpropanoïdes, se retrouve à des teneurs comparables chez les embryons des deux lignées. L'activité de la réductase du dihydroflavonol (DFR) qui pousse les phénols vers la synthèse de PA, est plus faible dans les téguments du YN01-429. Les résultats du transfert Southern et des analyses *in silico* indiquent la présence de deux copies du gène *DFR* chez le *B. napus*. Les deux copies sont fonctionnelles chez le YN01-429, ce qui élimine la répression ou le silencing homéoallélique, mais ensemble elles ne montrent que de très faibles degrés d'expression (transcriptions 17 fois inférieures) par rapport à l'activité du *DFR* chez les téguments du N89-53. Ces résultats pris dans leur ensemble suggèrent que le YN01-429 diffère à l'échelle des circuits qui agissent sur la ligne de synthèse des PA beaucoup plus que sur la ligne de synthèse des flavonols dans les téguments, et de tels circuits n'affectent pas le métabolisme général des phénylpropanoïdes chez les embryons.

Mots-clés : tannin condensé, réductase du dihydroflavonol, flavonoïdes, colza,oléagineux, colza, téguments transparents.

[Traduit par la Rédaction]

Introduction

Canola refers to lines of *Brassica napus* L. and *Brassica rapa* L. that have much reduced erucic acid (<2% in oil) and gluocosinolate contents (<30 μmol·g^{-1} oil-free solid) in the seeds. Worldwide, *B. napus* is the most commonly cultivated oilseed rape species. *Brassica napus* is a

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self-fertile allotetraploid containing the AA and CC genomes of *B. rapa* and *Brassica oleracea* L., respectively (Kimber and McGregor 1995). Although canola seed contains nearly 45% oil (*w*/*w*), it has approximately 20% protein (*w*/*w*) as well, and it leaves a protein-rich byproduct (~40% protein, *w*/*w*) after the oil has been extracted. Because of the amino acid, mineral, and vitamin content and composition, the defatted meal is a valuable animal feed supplement. However, the presence of antinutritional factors (ANF) detract from realizing the full value (Bell 1984,1993; Pusztai 1989). Fiber is one such ANF. Fiber, much of it contributed by the seed coat that constitutes nearly one-sixth of the seed mass, is not digestible by monogastric animals. The seed-coat-associated dark pigments of oxidized proanthocyanidins are also considered undesirable in the meal because of their interference with nutrient availability (Naczk et al. 1998; Lindeboom and Wanasundara 2007) and the speckled appearance of the meal containing those seed coat fragments.

The importance of the seed coat was recognized very early on in the use of *B. napus* seed meal as animal feed (Bell and Shires 1982; Shahidi and Naczk 1989). There are many ongoing efforts to breed a yellow-seeded *B. napus* that does not have dark pigments in the seed coat (Tang et al. 1997; Badani et al. 2006; Rahman 2001; Rahman et al. 2001). Such seeds are yellow because the underlying embryo is visible through a translucent seed coat. With the yellow-seed trait as a screening factor in breeding, it is conceivable that a thinner seed coat characteristic that reduces the opacity of the seed coat would have also been co-selected for in these efforts (Stringham et al. 1974; Badani et al. 2006). Yellow seeds have more oil because their seed coats are thinner and their embryos are larger (Abraham and Bhatia 1986), and their meal is also a better animal feed because of its relatively lower fiber and higher metabolizable energy (Slominski et al. 1994, 1999; Simbaya et al. 1995; Slominski 1997).

Because *B. napus* is an amphidiploid, it has been relatively difficult to breed for the yellow-seed trait. All common cultivars are of the dark-seeded type. Line YN01-429 of *B. napus* is the most advanced yellow seed germplasm of canola quality, arising from work spanning over three decades (Rakow and Relf-Eckstein 2005). While YN01-429 itself is a highly inbred (F_6) line, it is a derivative of complex intra- and inter-species genetic crosses. Reciprocal backcrossing with black-seeded lines followed by selfing does not give discrete yellow and black F_2 segregants, indicating involvement of many genes/alleles. In view of the paucity of information on the genetic and molecular genetic bases of the yellow-seed characteristic, we have investigated the phenolics content of the seed coat in YN01-429 and in a black-seeded relative with a view to understanding the underlying biochemical genetic basis of the trait. Here we report that in the yellow-seeded line there is a severe reduction in *DFR* (dihydroflavonol 4-reductase) gene expression and also in DFR enzyme activity that provides precursors for condensed tannins (Stafford 1990; Fig. 1). We also present evidence for the presence of two *DFR* genes in YN01-429 and in black-seeded lines and show that both are transcriptionally active albeit at a much lower level in YN01-429.

Material and methods

Plant material

Arabidopsis thaliana L. ecotype L*er* (hereinafter *Arabidopsis*) and *tt3*, *tt6*, *tt4*, and *tt7* mutant seeds in this genetic background were from the Arabidopsis Biological Resource Center (www.biosci.ohio-state.edu/pcmb/Facilities/abrc/abrchome.htm). *Brassica napus* lines YN01-429, N89-53, and DH12075 were kindly provided by Dr. Gerhard Rakow (Agriculture and Agri-Food Canada, Saskatoon). Strain N89-53 is the nearest black-seeded line in the complex ancestry of the yellow-seeded YN01-429 line (Rakow and Relf-Eckstein 2005). It was used as the black-seeded control line. Hereinafter, "black" and "yellow" lines will refer to these germplasms. These were grown under controlled growth conditions: temperature, 22 °C (day) – 16 °C (night); light intensity, 150–300 μmol quanta·m^{-2}·s^{-1}; light duration, 16 h. Flowers were labeled at anthesis, developing seeds were collected at various intervals, and mature and dry seeds were collected at 60 dpa. Manually removed seed coats were frozen in liquid nitrogen, lyophilized, and stored until further use.

Analysis of phenolics

Lyophilized seed coat tissue (20 mg) of *B. napus* or whole seeds of *Arabidopsis* were pulverized by vigorous shaking with zirconium beads (150 mg) and extacted with 500 μL of 70% acetone for 1 h at room temperature (22 °C) on a shaker. The supernatant, after centrifugation at 21 000*g* for 10 min, was retained. The total phenolic content was determined by the Folin–Ciocalteau assay as described by Singleton and Rossi (1965), coumaric acid was used as the standard. PA content in catechin equivalent of *B. napus* seed coat at different developmental stages was measured using a dimethylaminocinnamaldehyde (DMACA) assay, performed according to Li et al. (1996). For hydrolysis, acetone extracts from which the organic solvent had been removed under nitrogen flow were treated with a five-fold dilution of concentrated hydrochloric acid in water for 1 h at 90 °C. Aglycones were extracted with ethyl acetate, after which, the solvent was evaporated under a stream of nitrogen. Samples were dissolved in methanol and then subjected to HPLC analysis. All analyses were done in triplicate.

High performance liquid chromatography analysis

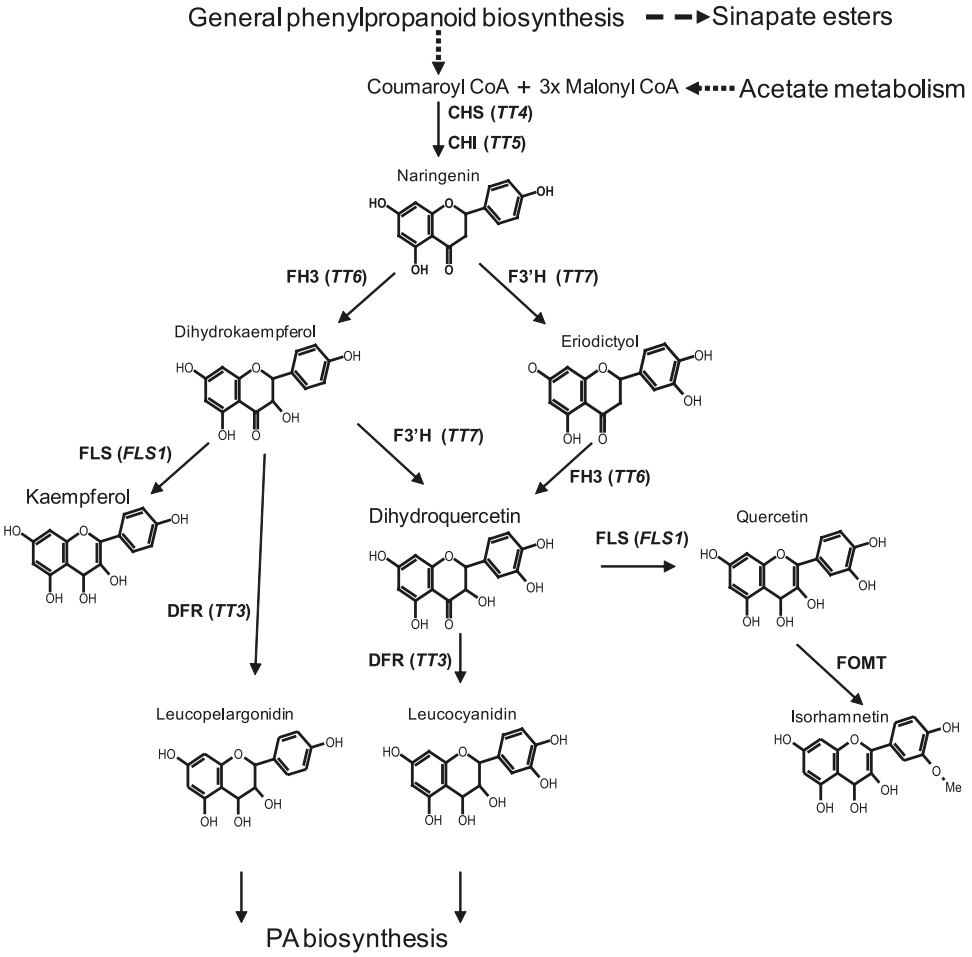
Flavonoids

HPLC was conducted with Waters system (Waters, Millford, Mass.), which comprised a 600 system controller, a 996 diode array detector, and a 717 Plus auto injector. A Waters Nova-Pak RP_{18} (5 μm, 3.9 mm × 150 mm) column with a solvent gradient of solvent A (1.5% H_3PO_4) and solvent B (acetonitrile) was used as follows. At 0 min, 80% A – 20% B; 25 min, 70% A – 30% B; 35 min, 30% A – 70% B; 40 min, 0% A – 100% B. Absorbance data at 360 nm were processed digitally with Millenium32 chromatography software. Known standards from Sigma-Aldrich (USA) were used alone and in co-chromatography. Flavonoid quantity is expressed in quercetin equivalents

Sinapine

Mature seeds were extracted with 75% ethanol at 22 °C

Fig. 1. Proanthocyanidin and flavonol synthesis pathways as known in *Arabidopsis*. Proanthocyanidins are formed from leucoanthocyanidins, the products of DFR, and also from flavan-3-ols derived from leucoanthocyanidins. CHS, chalcone synthase; CHI, chalcone isomerase; FH3, flavanone 3-hydroxylase; F3′H, flavonoid 3′ hydroxylase; DFR, dihydroflavonol 4-reductase; FOMT, flavonol 3′-*O*-methyltransferase; PA, proanthocyanidins. Where the genes are known, they are indicated in parentheses.



for 30 min, then centrifuged at 20 000*g* for 25 min. The solvent system comprised the following: solvent A (1.5% H_3PO_4) and solvent B (acetonitrile); a gradient pump operation program as follows. At 0 min, 100% A – 0% B; 5 min, 90% A – 10% B; 30 min, 85% A – 15% B. Sinapine was detected at 328 nm.

Protein extraction and enzyme assay

Dissected seed coats ground to a fine powder in liquid nitrogen were further homogenized (ground glass on glass) in the extraction buffer (50 mmol·L^{-1} Tris-HCl, pH 7.0, 100 mmol·L^{-1} KCl, 3 mmol·L^{-1} DTT, 0.4% PVPP (polyvinylpolypyrrolidone)). One tablet of Complete Mini EDTA-free Protease Inhibitor cocktail (Roche Diagnostics Canada, Laval, Que.) was used for 10 mL of buffer. The homogenate was centrifuged at 16 000*g* for 15 min at 4 °C, the supernatant was centrifuged again at 16 000*g* for 15 min at 4 °C. The resultant supernatant was assayed for enzyme activity after determining the protein concentration using a modified Bradford Protein Assay (Bio-Rad Laboratories, Mississauga, Ont.). The assay mixture contained in a total volume of 200 µL: 50 mmol·L^{-1} Tris-HCl, pH 7.5; 200 nmoles of DHQ; 0.66 µmol·L^{-1} of NADPH; and 200 µg of crude enzyme solution. The reaction, initiated by the addition of the crude enzyme solution, was allowed to proceed for 1 h at 28 °C and was stopped by vigorous mixing with ethyl acetate (600 µL). The ethyl acetate layer was washed with water and evaporated by nitrogen flow. The residue was re-dissolved in methanol and the PA content was determined using the DMACA assay. The PA content is expressed as catechin equivalents.

Southern blot analysis

Young leaf tissue was ground into a fine powder with liquid nitrogen and the DNA was extracted using the method described in Ausubel et al. (1989). Three micrograms of DNA was digested with restriction enzymes according to the methods of the supplier (New England Biolabs Inc.) and separated on a 0.8% agarose gel (1 × TAE: 40 mmol·L^{-1} Tris-base; 20 mmol·L^{-1} acetic acid; and 1 mmol·L^{-1} EDTA pH 8.0) for 18 h at 0.5 V·cm^{-1}, and Southern blotting was carried out using 0.4 N NaOH for 48 h. Full length genomic sequence of *DFR* amplified from *B. napus* DH12075 was labeled with ^{32}P (dCTP) using the Amersham Rediprime II Random Prime Labeling System (Amersham Biosciences, Piscataway, N.J.). Hybridization and washing were done us-

ing the QuickHyb System (Stratagene, La Jolla, Calif.) according to the manufacturer's instructions. Five millilitres of QuickHyb was used; prehybridization and hybridization were at 68 °C for 1 h, and the first two washes were with 2× SSC + 0.1% SDS for 15 min each, and the next two washes were with 0.2× SSC + 0.1% SDS, 15 min each, at 65 °C. The blots were exposed to a Phosphor Storage Screen overnight and scanned with a Storm 840 scanner (Amersham Biosciences). Images were analyzed with ImageQuant version 5.2 (Molecular Dynamics, Sunnyvale, Calif.). For amplifying the genomic region of *DFR* that was used for making probes, primers based on consensus sequences from *DFR* ESTs and *Arabidopsis DFR* sequences were used.

Quantitative reverse-transcriptase polymerase chain reaction

Dissected seed coats were ground to a fine powder in liquid nitrogen and total RNA was extracted using an RNeasy Plant Mini Kit (QIAGEN, Mississauga, Ont.) according to the manufacturer's instructions. First-strand cDNA was synthesized using SuperScript™ III Reverse Transcriptase (Invitrogen, Burlington, Ont.). Quantitative reverse-transcriptase PCR (qRT-PCR) was carried out on an Mx3000P QPCR System with MxPro QPCR Software (Stratagene, La Jolla, Calif.) using a QuantiTect SYBR Green RT-PCR Kit (QIAGEN) according to manufacturer's instructions. The qRT-PCR reaction was initiated with incubation at 95 °C for 15 min followed by 40 cycles of 95 °C for 30 s; 58 °C for 45 s; and 72 °C for 30 s. Three biological replicates with two technical replicates of each biological replicate were analyzed. Relative changes in gene expression were calculated using the $2^{-\Delta\Delta C_T}$ method described by Livak and Schmittgen (2001) with the 25 dpa sample of the black line (N89-53) as the calibrator and actin for normalization. Quantitative PCR primers were as follows: Actin forward (OL-5394) 5′-AACCCAAAGGCCAACAGAGA-3′; Actin reverse (OL-5395) 5′-AAGGTCACGTCCAGCAAGGT-3′; DFR forward (OL-6673) 5′-TATGCCGCCTAGCCTTATTACCG-3′; DFR reverse (OL-6674) 5′-CCTTGGCAGCAGCTTGTTCGT-3′.

Determination of allelic expression

This was based on the diagnostic method of Konieczny and Ausubel (1993) that was originally described for mapping genomic polymorphisms. The coding sequence of *DFR* (from the 2nd codon) was amplified from cDNA preparations of 25 dpa seed coats from black and yellow seed lines. After amplification, aliquots of the amplicons and their *Bam*HI-digests (done according to supplier's instructions) were electrophoresed on a 1% agarose gel containing GelRed™ Nucleic Acid Gel Stain (Biotium, Hayward, Calif.). The PCR conditions were 94 °C incubation for 2 min, followed by 40 cycles of 95 °C for 60 s; 56 °C for 60 s; and 72 °C for 60 s; then extension at 72 °C for 10 min. Three independent tissue samples were used.

Results

We have performed a comparative analysis of the proanthocyanidin content in the seed coats of the yellow-seeded *B. napus* line YN01-429 and a related black-seeded line N89-53. Under our experimental conditions, the seeds became mature and dry by 60 dpa in both lines. Young developing seeds were green in both lines up to ~40 dpa. Thereafter in the black line, but not the yellow line, the seeds started to turn brown before becoming progressively darker owing to oxidized PA.

Phenolics biosynthesis is perturbed in the seed coat but not in the embryo of the yellow-seed canola.

Our initial experiments had indicated that the total amount of extractable phenolics in seed coats from mature seeds was very low. To determine at what point during development the black and yellow lines begin to deviate in phenolics accumulation, we assayed isolated seed coats from seeds at various stages of development. As shown in Fig. 2, phenolics were evident at 10 dpa in both lines. In the black line, the phenolics content increased substantially over the next 10 d, and remained nearly at the same level until 40 dpa before the level of extractable phenolics declined in mature seeds. However, in the yellow line, at 10 dpa, the amount of phenolics was ~50% relative to the black line, but in contrast to the latter, the level did not increase as the seeds advanced to 40 dpa. Since the Folin–Ciocalteau assay measures all phenolics, these results show a general impairment in phenolics biosynthesis in the seed coat tissue from YN01-429. To determine whether this is the case for other tissues in the seed, we measured the sinapine content of whole seeds as well; sinapine is one of the terminal products of general phenylpropanoid biosynthesis in crucifers and it accumulates in seed embryos (Nair et al. 2000). The sinapine content of YN01–429 (6 ± 0.4 $mg \cdot g^{-1}$) was similar to that of N89-83 (7 ± 0.6 $mg \cdot g^{-1}$) showing that phenolics metabolism was not perturbed in the seed.

PA synthesis is severely diminished in the seed coats of the yellow seed but not the black seed line

Oxidized PA is visible as brown-black pigmentation of seed coats (Pourcel et al. 2006). We assayed hydrolyzed aliquots of seed coat extracts for PA (Fig. 3). The PA content was low at 10 dpa in the black seed line and it was barely detectable in the yellow line. As in the case of the total phenolic content, there was a rapid increase of extractable PA in the black line at 20 dpa, followed by a decline from 30 dpa onwards presumably due to a higher degree of polymerization that negatively affects extraction. In contrast, the seed coat from the yellow-seeded line contained ~100-fold less PA when compared with seed coat from the black-seeded line and there was no developmental stage-specific increase in extractable PA.

The flavonol biosynthesis branch is relatively less affected in the yellow line

We could not detect chalcones, the very early substrates in the pathway to PA synthesis, or the immediate precursors such as flavan-3-ols in the seed coats of either seed type, presumably due to rapid channeling/conversion of these substrates in the metabolic pathway (Fig. 1). However, kaempferol, quercetin, and 3′*O*-methylated quercetin (isorhamnetin) that share precursors with PA were found in both seed types. Unlike the 100-fold reduction in PA content, the seed coat of the yellow line at 60 dpa contained as much as half (0.04 ± 0.004 $\mu g \cdot mg^{-1}$) of the flavonol content (kaempferol + quercetin + isorhamnetin) of the seed coat from the black line (0.08 ± 0.001 $\mu g \cdot mg^{-1}$) (Fig. 4). The dif-

Fig. 2. Total phenolics in seed coats dissected from developing seeds of black (N89–53) and yellow (YN01–429) lines of *Brassica napus*. At 60 dpa, the seeds were mature and dry.

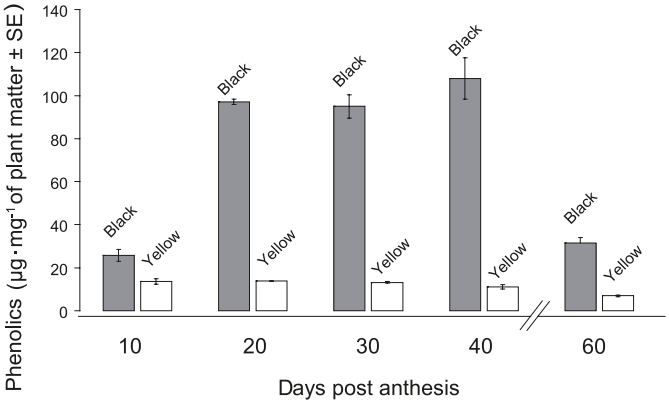
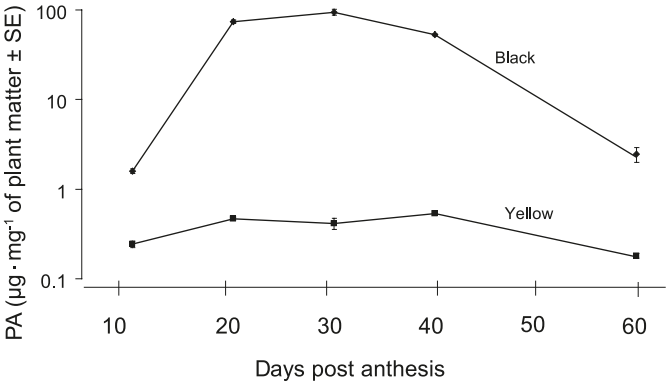


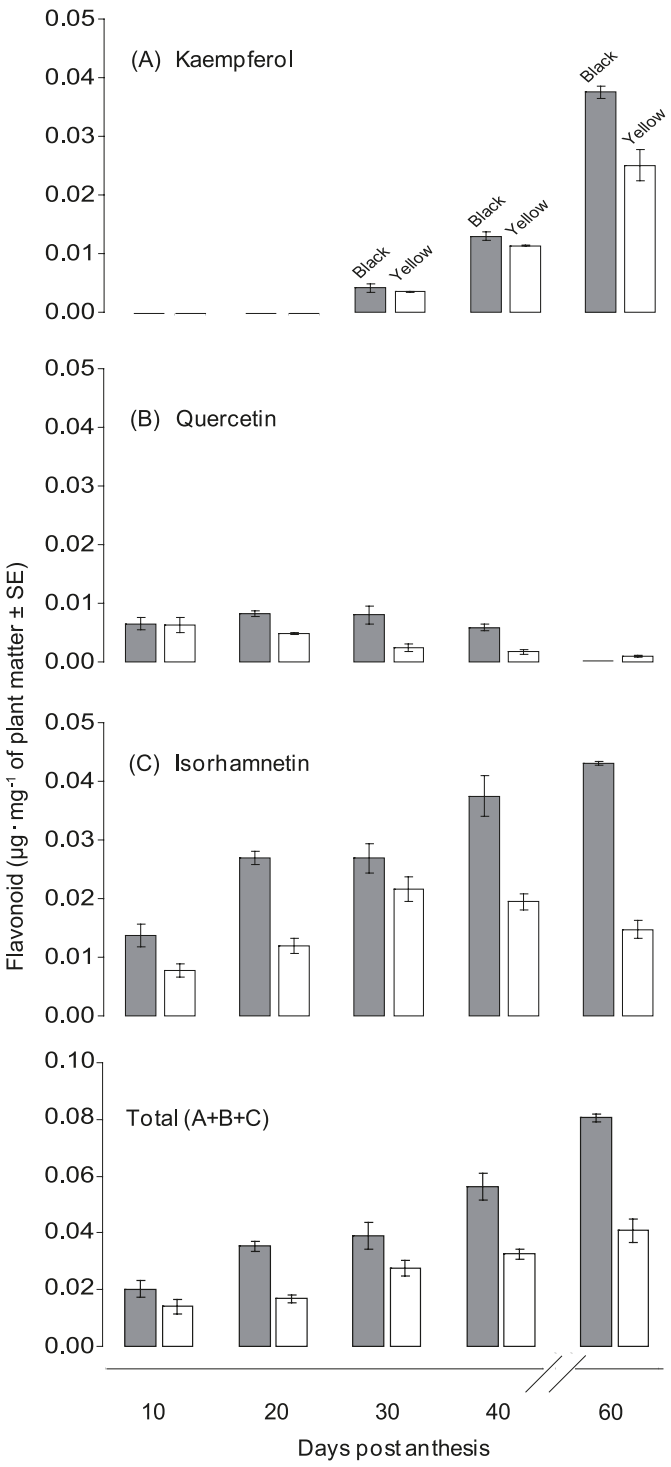
Fig. 3. Total proanthocyanidins in seed coats dissected from developing seeds of black (N89-53) and yellow (YN01-429) lines of *Brassica napus*. At 60 dpa, the seeds were mature and dry.



ference was even less in younger stages, particularly at 30 dpa (0.039 ± 0.005 µg·mg^{-1} for the black- and 0.027 ± 0.003 µg·mg^{-1} for the yellow line). This clearly shows that PA synthesis is severely constrained while flavonol biosynthesis is only relatively mildly perturbed in the seed coats of the yellow seeds. Since DMACA would react with PA as well as the precursors flavan-3-ols and flavan-3,4-diols, the results collectively suggested a defect in the dihydroflavonol 4-reductase (DFR)-catalyzed step that produces leucoanthocyanidins (flavan-3,4-diols).

Taking advantage of the genetic lesions and resultant alterations in the flavonoid composition of *A. thaliana* mutants, we compared the flavonol and anthocyanidin composition of selected *tt* mutants of *Arabidopsis* with that of the two *Brassica* lines. Of the four mutants, only *tt3* (DFR defective) and not *tt4*, *tt6* nor *tt7* was similar to YN01-429 (Fig. 5). Note that quercetin is present in *tt3* and its *O*-methylated form (isorhamnetin) is present in YN01-429. Although *Arabidopsis* has been found to produce a quercetin *O*-methyl transferase (Muzac et al. 2000), the production of isorhamnetin does not appear to be as robust as in *Brassica*. Although YN01-429 resembled all four *tt* mutants in terms of having very little PA, the presence of flavonols in YN01-429 distinguishes it from *tt4* and *tt6*. The presence of 3-hydroxylated flavonols in YN01-429 rules out a defective flavonone 3-hydroxylase (*TT7* gene product).

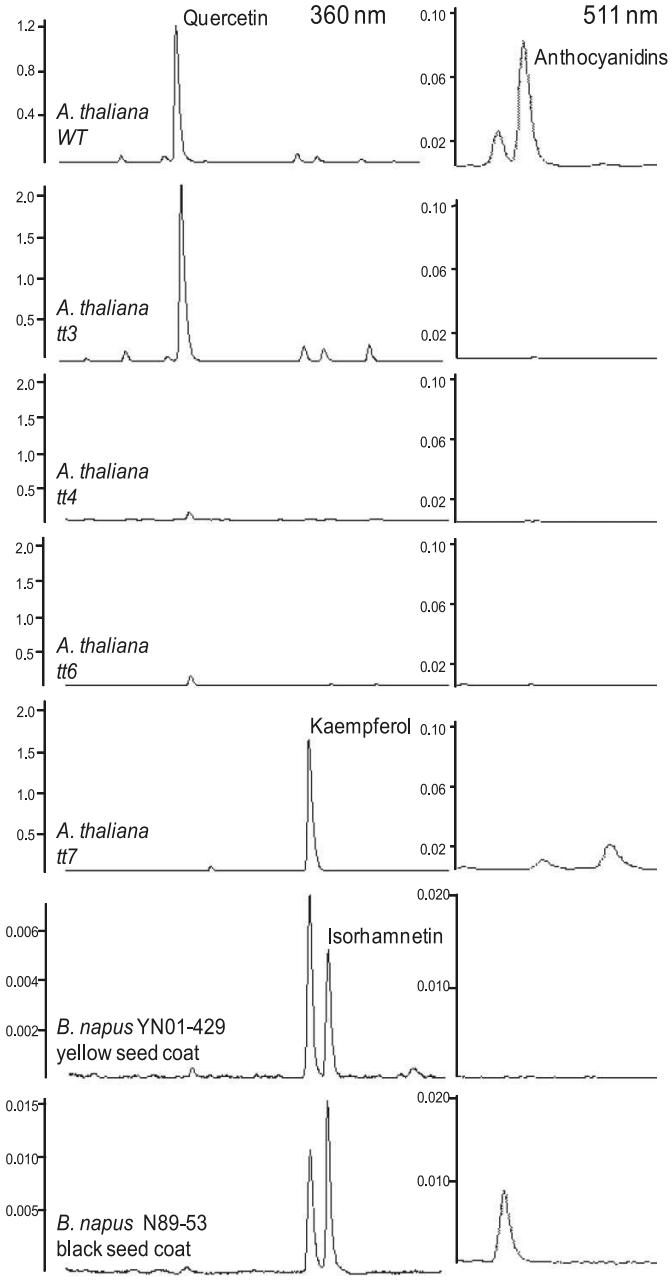
Fig. 4. Kaempferol, quercetin, isorhamnetin, and total flavonols in seed coats dissected from developing seeds of black (N89-53) and yellow (YN01-429) lines of *B. napus*. At 60 dpa, the seeds were dry.



Seed coats of the yellow seed line have a very low level of DFR enzyme activity, likely owing to lower levels of gene expression

We assayed DFR enzyme activity in comparably prepared crude extracts from seed coats of both seed types. In the black-seeded line, 20 dpa seed coats showed the highest ac-

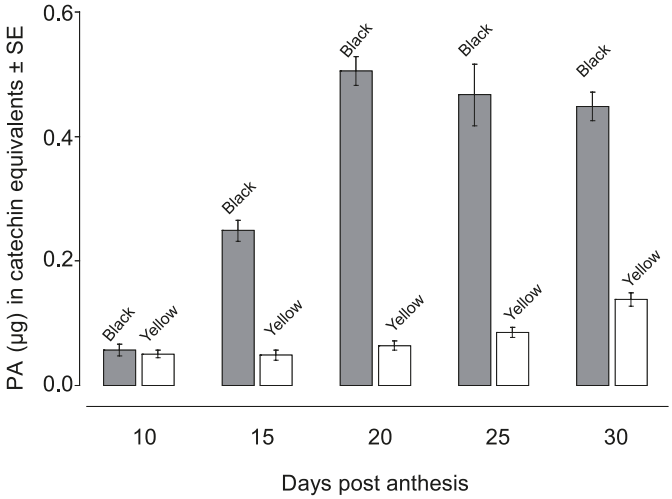
Fig. 5. HPLC profiles of hydrolyzed acetone extracts from mature seeds of *Arabidopsis thaliana* L*er* (shown as *WT*) and transparent testa mutants (*tt*) in this background, and 40 dpa seed coat dissected from black (N89-53) and yellow (YN01-429) lines of *B. napus*. Note that individual HPLC profiles are not given at the same scale (in absorbance units). Absorbance was measured at 360 nm (flavonols) and 511 nm (anthocyanidins).



tivity while the yellow seed line had ~10-fold less activity (Fig. 6). The relative activity in the seed coats of the yellow line was lower at all stages of development that were examined, with the exception of 10 dpa when both lines had low activity. These experiments show that at the most robust stage of PA synthesis, i.e., 20 dpa (recall from Fig. 3), the seed-coat tissue in the yellow line has much less capacity to convert dihydroflavonols to PA precursors.

We investigated whether the low level of DFR activity

Fig. 6. Dihydroflavonol 4-reductase (DFR) activity of crude protein extracts of seed coats dissected from developing seeds of black (N89-53) and yellow (YN01-429) lines of *Brassica napus*. Catechin standard curve was used for measuring the DFR assay product.



was due to reduced gene expression in the seed coats of YN01-429 seeds (Fig. 7). This was indeed the case: qRT-PCR analysis showed that the YN01-429 seed coat had less *DFR* transcripts at all stages of development up to 25 dpa. At 20 dpa, which is a critical window for PA accumulation, YN01-429 had 17-fold less transcript relative to N89-53. The temporal pattern of gene expression was in agreement with that of enzyme activity and also PA accumulation in black seed coats, considering that PA accumulation would be cumulative.

Lower *DFR* expression is not due to loss of one or more *DFR* genes in the yellow-seeded line

Some phenylpropanoid–flavonoid biosynthesis genes occur as multigenic families (Lagercrantz. 1998; Chen et al. 2007). The copy number of *DFR* in *B. napus* has not been reported. It is also known that the genomic organization of some homeologous gene families change in polyploids affecting the expression or even total loss of some genes (Chen and Ni 2006). We estimated the copy number of *DFR* in YN01-429, N89-53, and DH12075 (a black-seeded dihaploid line), by Southern blot analysis. The data presented in Fig. 8 suggest two *DFR* copies in all three lines: Only two strongly hybridizing bands were seen when the genomic DNA was cut with *Eco*RV for which there is no site within the *DFR* gene. With *Hin*dIII, three bands were obtained because of an internal site in one of the DFR genes. These experiments also show that one of the two *DFR* genes is in a region that is polymorphic in YN01–429 with reference to the two black-seeded lines.

The inference of two DFR genes is also indirectly supported by analysis of *DFR* ESTs reported in GenBank (30 May 2008) that include seed coat ESTs generated in our laboratory. From 107 908 seed coat ESTs, 20 ESTs that nearly match *B. rapa DFR* cDNA (gi|61699137) (E-value between 0 and 5E-64) were found. Fifteen of these could be organized into Type 1 (3 ESTs) and Type 2 (12 ESTs) based on sequence alignment; the remaining 5 were shorter sequences that could not be differentiated. The two groups were polymorphic at 15 dispersed nt positions; 8 of these were within

Fig. 7. Quantitative PCR assay of *DFR* expression. Seed coats dissected from developing seeds of black (N89-53) and yellow (YN01-429) lines of *Brassica napus* were used, and the expression value for the 25 dpa sample of the black line was chosen arbitrarily as the calibrator.

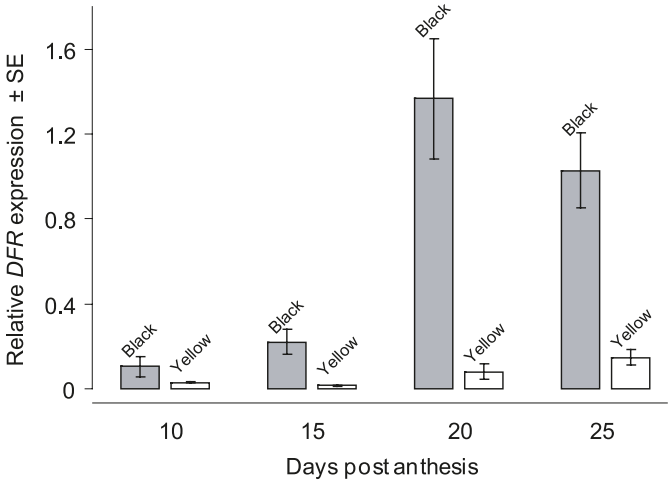
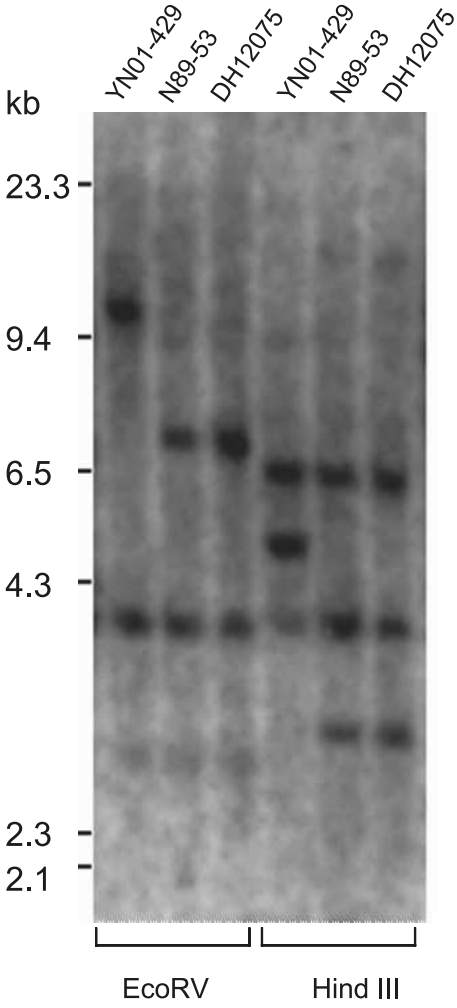


Fig. 8. Southern blot analysis of genomic DNA. PCR-amplified genomic DNA of *Brassica napus* with primers at the two extreme ends of the deduced ORF was used for probing. In separate analyses, one of the clones of the amplicons was found to have one site for *Hin*dIII but none for *Eco*RV. Another independent clone was nearly identical but did not have a site for either enzyme. The extra band in the *Hin*dIII-digests is considered to arise from cleavage within a homeologous *DFR* gene of the amphidiploid genome. The hybridization wash conditions were of medium stringency.



the ORF but did not alter the deduced amino acid sequence. Among all 20 ESTs, only one was from YN01-429 seed coat (27 139 ESTs) and it belonged to Type 2. An additional set of 23 ESTs were also present in the remainder of the *B. napus* ESTs at GenBank and these were also from seed tissue. A lone seedling EST with a 67-nt deletion was also found and it was identical to the Type 2 EST except for the deletion. Excluding this, the 42 ESTs were all from whole seed or seed coat tissue and they belonged to only the two types noted above (Type 1, 16 ESTs; Type 2 19 ESTs; 7 shorter ones of either type). Accessions EV041488 and EV042831 represent the longest of Type 1 and Type 2, respectively.

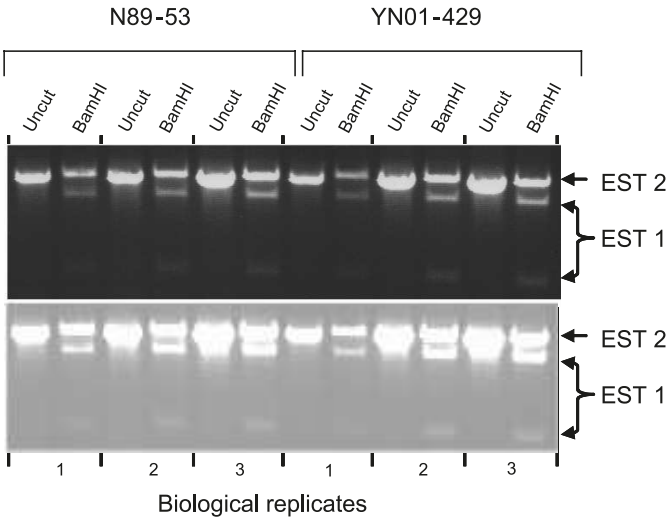
Low level of DFR expression in YN01-429 is not due to selective silencing of one of the two gene copies.

Silencing of some homeoalleles occurs in polyploids (Chen and Ni 2006). Although only one yellow seed EST was present in the above EST collection, we found that the second gene was also active in RT-PCR assays. An SNP was found at position 282 of the ORF of Type 2 EST that removes a BamH1 site present in Type 1 EST. We exploited this feature to determine allelic expression. As shown in Fig. 9, YN01-429 seed coat tissue expresses both genes. There was also no indication of Type 1 being expressed to a lesser extent than Type 2 in YN01-429.

Discussion

Canola seed meal contains fiber, phenolics, and pigments from seed coats because this is not removed prior to oil extraction. This detracts from realizing a better economic value of the protein-enriched meal. Unpigmented seed coats would mitigate this problem to some extent, and in this regard YN01-429 presents an opportunity to breed such elite canola varieties. This line had not been characterized genetically or biochemically to identify the bases for its translucent–transparent seed coat. Our comparison shows that PA accumulates in the seed coats of the control black line very rapidly between 10 and 20 d post anthesis and that *DFR* activity is also at its maximum at this time. We have shown previously that the inner integument develops and differentiates rapidly at young stages of seed development before it undergoes programmed cell death and eventually appears as a pigment layer between the aleurone and outer integument (Wan et al. 2002). Though these cytological investigations were done with a different cultivar (*B. napus* 'Westar'), the temporal aspects of PA and total phenolics accumulation reported in the present study are in general agreement. PA accumulation is, however, only barely detectable in the yellow-seeded line; *DFR* expression is severely affected and the output of general phenylpropanoid metabolism is also reduced in the seed coats. Interestingly this is not the case with the embryo. *Brassica napus* appears to have two homeoalleles of *DFR* and both are present and transcribed in the yellow-seeded line. All these point to an overall depression of phenolics biosynthesis

Fig. 9. Assay for expression of *DFR* alleles in seed coat tissue. Reverse transcription reaction products with RNA from 25 dpa seed coats of *Brassica napus* were PCR-amplified with primers situated near the extreme ends of the *DFR* open reading frame (see Materials and methods). The primers were not allele-specific. The amplicons were cut with *Bam*HI (known to be present in Type 1 but not Type 2 EST) to differentiate allelic expression. The bottom panel is a digital "overexposure" of the upper panel to show the smaller band from *Bam*HI cleavage.



and transcriptional downregulation of *DFR* specifically in seed coat tissues. Additional experiments are required to identify other specific blocks in the phenolics metabolism. The severe constraint in *DFR* production nearly eliminates the flow of dihydroflavonols through the procyanidin/anthocyanidin branch. Unlike the situation with *tt3* mutant of *Arabidopsis* where the quercetin level increases about two-fold relative to wild-type seeds, there is no such increase in the flavonol content of the seed coats from the yellow line. In fact, the level of flavonoids in the yellow-seeded line is two-fold less when compared with the black-seeded line. This suggests that, besides the major obstruction of the PA pathway at the level of DFR step, there are other, less severe impediments to flavonoid biosynthesis in the yellow line.

While the genetics of flavonoid metabolism was elucidated originally in maize, barley, petunia, and *Antirrhinum*, the *transparent testa* mutants of *Arabidopsis* have been very valuable in understanding the genetic circuitry (Winkel-Shirley 2001; Grotewold 2004; Lepiniec et al. 2006; Pourcel et al. 2006). A panel of *Arabidopsis tt* mutants was useful in comparative flavonoid profiling to identify the defects in the canola line. There are 21 non-allelic *tt* mutations of which 6 correspond to regulatory genes (Lepiniec et al. 2006). Of the latter, three (*TTG1*, *TT2*, *TT8*) impact *DFR* expression. *TTG1* and *TT8* are expressed in vegetative and seed tissue whereas the expression of *TT2* is limited to the endothelium. These *TT* genes encode, respectively, WD-R, MYB and bHLH types of transcriptional factors, which take part in the regulation *DFR* and *LDOX* (Lepiniec et al. 2006). *B. napus TT2* genes have been cloned; a yellow-seed germplasm developed in China does not show less expression of *TT2* genes (Wei et al. 2007).

Extensive chemical analysis of other black seeded *B. napus* lines (Durkee 1971; Leung et al. 1979; Mitaru et al. 1982; Naczk et al. 1998) and elucidation of flavonoid biochemistry in general (Marles et al. 2003*a*; Marles and Gruber 2004) have provided a sound basis to apply metabolite profiling to probe the genetic causes of yellow seed trait. For example, Marles et al. (2003*b*) compared a yellow-seeded and a brown-seeded type found in a seed lot of *Brassica carinata* (BB CC genome) after selfing the lines and showed that the yellow seed had lower levels of *DFR* expression. *B. carinata* shares a C genome with *B. napus* but it is not known if the above-mentioned line was used in developingYN01-429. Regardless, it will be interesting to determine if the RFLP of the DFR-containing genomic region of YN01-429 noted in our study also occurs in *B. carinata*

In conclusion, our results show that low *DFR* expression is one of the contributing factors to the yellow seed trait in YN01-429. Our results further show that this is not the only determining factor since the production of phenolics in the seed coat in general is also impaired, presumably owing to altered transcriptional regulation. Unraveling the latter would be useful for obtaining molecular handles for facile introduction of the economically important yellow seed trait into otherwise elite dark-seeded *B. napus* varieties. This is particularly pertinent because essentially all cultivated *Brassica napus* is of the dark-seeded type and there is an ongoing global effort to develop yellow-seeded varieties.

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