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RESEARCH PAPER

Differential accumulation of phenolic compounds and expression of related genes in black- and yellow-seeded *Brassica napus*

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Abstract

Developing yellow-seeded *Brassica napus* (rapeseed) with improved qualities is a major breeding goal. The intermediate and final metabolites of the phenylpropanoid and flavonoid pathways affect not only oil quality but also seed coat colour of *B. napus*. Here, the accumulation of phenolic compounds was analysed in the seed coats of black-seeded (ZY821) and yellow-seeded (GH06) *B. napus*. Using toluidine blue O staining and liquid chromatography–mass spectrometry, histochemical and biochemical differences were identified in the accumulation of phenolic compounds between ZY821 and GH06. Two and 13 unique flavonol derivatives were detected in ZY821 and GH06, respectively. Quantitative real-time PCR analysis revealed significant differences between ZY821 and GH06 in the expression of common phenylpropanoid biosynthetic genes (*BnPAL* and *BnC4H*), common flavonoid biosynthetic genes (*BnTT4* and *BnTT6*), anthocyanin- and proanthocyandin-specific genes (*BnTT3* and *BnTT18*), proanthocyandin-specific genes (*BnTT12*, *BnTT10*, and *BnUGT2*) and three transcription factor genes (*BnTTG1*, *BnTTG2*, and *BnTT8*) that function in the flavonoid biosynthetic pathway. These data provide insight into pigment accumulation in *B. napus*, and serve as a useful resource for researchers analysing the formation of seed coat colour and the underlying regulatory mechanisms in *B. napus*.

Key words: *Brassica napus*, expression profile, flavonoid pathway, phenolic compounds, regulatory mechanism, seed coat colour.

Introduction

Brassica napus (2*n*=38, AACC) is one of the most important oilseed crops worldwide. Increasing oil yield is the foremost objective of *B. napus* breeding programmes. In *B. napus*, the oil yield is simultaneously determined by seed yield and oil content. Much effort has focused on identifying a germplasm and hybrid cross with higher seed oil content. A remarkable advancement was the development of yellow-seeded *B. napus*, which has a significantly thinner seed coat, lower husk proportion, higher oil content, and much lower crude oil pigment content than the black-seeded type (Olsson, 1960; Stringam *et al.*, 1974; Tang *et al.*, 1997; Meng *et al.*, 1998). However, the genetic mechanism underlying seed coat colour formation in *B. napus* is not well understood, and 1–4 genes are thought to be involved in this process (Somers *et al.*, 2001; Liu *et al.*, 2005; Xiao *et al.*, 2007; Zhang *et al.*, 2011).

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The seed coat colour of *B. napus* is determined by the content of phenolic compounds cyanidin and procyanidins (Marles and Gruber, 2004; Akhov *et al.*, 2009; Nesi *et al.*, 2009). Flavonoids possess diverse chemical structures with a common C_6-C_3-C_6 body. According to the linkage position of the aromatic ring, the saturation level, and the oxidation degree of the C-ring, flavonoids are classified into flavonols, anthocyanins, phlobaphenes, isoflavones, and proanthocyanidins (PAs) (Winkel-Shirley, 2001). Both cyanidin and procyanidins are phenolic compounds belonging to the PA class of flavonoids. In *B. napus*, the coats of the black-seeded variety have a higher flavonol and procyanidin content than do those of the yellow-seeded variety (Akhov *et al.*, 2009). In mature *B. napus* seeds, the coat is usually dark brown to black (Fu *et al.*, 2007). A previous study reported that the seed coats of *B. napus* accumulate high amounts of insoluble PAs (in-PAs), which are difficult to quantify. Auger *et al.* (2010) further demonstrated that soluble PA starts to accumulate early in embryogenesis in the micropyle–chalaza area and continues to collect during mid to late embryogenesis in the seed body. The flavonols present in *Brassica* seeds are kaempferol derivatives, quercetin derivatives, isorhamnetin derivatives, and epicatechin glucoside, and flavonols were also detected in the vegetative parts and inflorescences of *Brassica* spp. (Romani *et al.*, 2006; Lin and Harnly, 2009, 2010).

The phenylpropanoid and flavonoid pathway synthesizes a large array of secondary metabolites and has now been well characterized by the identification and functional analysis of several *Arabidopsis thaliana* mutants (Winkel-Shirley, 2002; Bharti and Khurana, 2003; Gachon *et al.*, 2005). Research in *Arabidopsis* has shown that the pathway starts with the conversion of L-phenylalanine into *trans*-cinnamic acid by phenylalanine ammonia lyase (PAL) (Supplementary Fig. S1 available at *JXB* online). *Trans*-cinnamic acid is hydroxylated by cinnamate 4-hydroxylase (C4H) to form *p*-coumaric acid (Russell, 1971), and 4-coumarate:CoA ligase (4CL) then converts *p*-coumaric acid into its coenzyme-A ester, which is the precursor of various phenylpropanoid derivatives, including flavonoids, lignins, and isoflavonoids (Winkel-Shirley, 2002). The formation of tetrahydroxychalcone, the precursor of all flavonoids, is catalysed by chalcone synthase (CHS), which is encoded by *TRANSPARENT TESTA 4* (*TT4*) (Burbulis and Winkel-Shirley, 1999). This is the first dedicated step of flavonoid biosynthesis in higher plants, and is thus critical for many important flavonoid-related characteristics, such as seed coat colour, flower colour, and pigmentation of the stem and leaf surface (Hoffmann *et al.*, 2006; Kasai *et al.*, 2007). Chalcone is converted into naringenin by chalcone isomerase (CHI), which is encoded by *TT5*. Subsequently, naringenin is converted into dihydrokaempferol and dihydroquercetin by flavone 3-hydroxylase (F3H; also known as TT6) and flavone 3'-hydroxylase (F3'H; also known as TT7) (Holton and Cornish, 1995; Pelletier and Shirley, 1996; Wisman *et al.*, 1998; Schoenbohm *et al.*, 2000; Grotewold, 2006). Then, the products are further metabolized via two biosynthetic branches (Supplementary Fig. S1). On one branch, dihydrokaempferol and dihydroquercetin are converted into leucocyanidin and leucopelargonidin, respectively, by DFR (TT3). Leucoanthocyanidin dioxygenase (LODX) then catalyses the conversion of leucocyanidin and leucopelargonidin into cyanidin and pelargonidin, respectively (Abrahams *et al.*, 2003). In the last step, cyanidin is converted into epicatechin by anthocyanidin reductase (ANR; also called BAN). On the other branch, dihydrokaempferol and dihydroquercetin are converted by flavonol synthase (FLS) to flavonols, such as kaempferol and quercetin. Quercetin can be converted into isorhamnetin by *O*-methyl transferase (OMT). These products are believed to be synthesized in the cytoplasm and transported to the vacuole by some transporters, such as TT12 (Marinova *et al.*, 2007; Zhao and Dixon, 2009). In addition, *TT19* (Sun *et al.*, 2012) was shown to encode a GST (glutathione *S*-transferase) protein related to flavonoid transport to the vacuole. Kaempferol, quercetin, and isorhamnetin are colourless or pale yellow, while cyanidin and pelargonidin are pink-red in the cytoplasm and pink-purple in the vacuole. Epicatechin units are polymerized to procyanidin oligomers and then oxidized to form insoluble procyanidins by TT10 (Pourcel *et al.*, 2005; Zhao *et al.*, 2010). *BAN* encodes one anthocyanidin reductase and is involved in plant flavonoid biosynthesis (Albert *et al.*, 1997; Devic *et al.*, 1999; Xie *et al.*, 2003; Baudry *et al.*, 2004). The expression of the underlying biosynthetic genes is regulated by a set of transcription factors and regulatory genes, such as *TT2*, *TT8*, *TT1*, *TTG1*, and *TTG2* (Nesi *et al.*, 2000, 2001; Chiu *et al.*, 2010).

In this study, cDNAs for phenylpropanoid biosynthetic genes (*PAL*, *C4HA*, and *C4HB*), flavonoid biosynthetic genes (*CHS*, *CHI*, *F3H*, *F3'H*, *DFR*, *LDOX*, *LAR*, *TT12*, *BAN*, and *TT10*), and regulatory genes (*TT2*, *TTG1*, *TTG2*, and *TT8*) were isolated from the seeds of *B. napus*. Differential transcriptional profiles of these genes in the black-seeded coat parent ZY821 and the yellow-seeded coat parent GH06 were analysed by quantitative real-time PCR (qRT-PCR) at seven different seed developmental stages. In parallel, the spatiotemporal accumulation of polyphenol compounds in different development stages of ZY821 and GH06 was analysed using toluidine blue O (TBO). Moreover, the accumulation of flavonols, PAs, and in-PAs in the seed coat was also quantified by HPLC-DAD-MS (high-performance liquid chromatography with diode-array detection and mass spectrometry). The results suggest that the differential expression of flavonoid biosynthetic genes plays a crucial role in determining the seed coat pigmentation patterns of black- and yellow-seeded *B. napus*.

Materials and methods

Plant materials and growth conditions

Inbred lines of GH06 and ZY821 were used to represent typical yellow- and black-seeded genotypes of *B. napus*, respectively. ZY821 is a major variety of rapeseed that has been cultivated in China since the 1980s and has a 39.69% oil content, 29.81% protein content, and 19.75% fibre content (Supplementary Fig. S2A, C, E at *JXB* online). GH06 is a yellow-seeded rape that is used as one of the breeding parents in the Chongqing Rapeseed Technology Research Center (CRTRC) and has a 44.57% oil content, 33.65% protein content, and 14.57% fibre content (Supplementary Fig. S2B, D, F). The plants were grown under normal field conditions at the CRTRC

in 2010. Field management essentially followed normal agronomic procedures. Seeds of the two parental lines (i.e. GH06 and ZY821) were harvested and used for total RNA isolation at seven developmental stages, namely 7, 14, 21, 28, 35, 42, and 49 days after pollination (DAP).

Tissue preparation and light microscopy observations

Brassica napus seeds were harvested at 7, 14, 21, 28, 35, 42, and 49 DAP and immediately fixed for 24 h at 4 °C in a fixation solution containing 5% acetic acid, 5% formaldehyde, and 50% ethanol. Following fixation, seeds were dehydrated at 60 min intervals through a 20% step-graded series of ethanol–water mixtures, ending at 100% ethanol. Then, the seeds were processed at 60 min intevals through a 30% step-graded series of ethanol–TBA (tert-butyl alcohol) mixtures, ending at 100% TBA. Seeds were subsequently infiltrated over a 24 h period with saturated paraffin–TBA mixtures, and then embedded over a 48 h period in paraffin. Blocks were completely polymerized at 4 °C.

Semi-thin (5–8 μm thick) sections were cut with a microtome blade R-35 (Feather Safety Razor Co., Ltd Medical Division, Japan) and viewed under a stereo microscope (SZX12, Olympus, Japan). Three blocks were sectioned for each time point, and a minimum of 60 sections were collected for each block. Sections were stained with TBO and observed with a Nikon Eclipse E600 microscope (Nikon Instruments, Japan).

Reagents and standards

Liquid chromatography–mass spectrometry (LC-MS) solvents were from Fisher Scientific (Rockford, IL, USA); ultra-pure water was obtained using a model MilliQ Plus system from Millipore (Billerica, MA, USA). Flavonoid standards were from Indofine (Somerville, NJ, USA), Sigma-Aldrich (St Louis, MO, USA), and Chromax (Irvine, CA, USA).

Extraction of flavonoids from seed coats

Frozen fresh seed material (100 mg fresh weight) was homogenized in 80% methanol (1 ml), and the suspension was placed in an ultrasonic bath for 1 h. The extract was centrifuged (13 000 rpm, 20 min) and the supernatant was filtered. The samples were immediately subjected to LC-UV-MS analysis. The in-PA measurement method was followed as previously described by Liang *et al.* (2006).

High-performance liquid chromatography analysis

Plant extracts were analysed with an Agilent 1100 HPLC system (Hewlett-Packard, Palo Alto, CA, USA) combined with an iron trap mass spectrometer and a Bruker Esquire 3000 (Bruker Daltonics, Bremen Germany). Instrument analyses were carried out using a Grace column (20 × 250 mm, grain diameter 4.6 μm). UV spectra were obtained by scanning from 200 nm to 600 nm. The mobile phase consisted of (A) water containing 0.1% formic acid (v/v) and (B) acetonitrile, using the following binary gradient: 0–5 min, isocratic 95% A and 5% B; 5–10 min, isocratic 10% B; 10–17 min, isocratic 17% B; 17–25 min, isocratic 25% B; 25–30 min, isocratic 30% B; 30–55 min, isocratic 55% B; 55–65 min, isocratic 70% B; 65–70 min, isocratic 5% B; and 70–75 min, isocratic 95% A and 5% B. The flow rate was 0.8 ml min^{-1} and the temperature of the column was maintained at 25 °C. Negative-ion electrospray ionization (ESI) mass spectra was employed, using an ion source voltage of 3.5 kV, a counter current nitrogen flow set at a pressure of 12 psi, and a capillary temperature of 350 °C. Mass spectra were recorded over the range 50–2200 *m*/*z*. The Bruker ion-trap mass spectrometer (ITMS) was operated using an ion current control (ICC) of ~10 000 with a maximum acquisition time of 100 ms. Tandem mass spectra were obtained in manual mode for targeted masses using an isolation width of 2.0, fragmentation amplitude of 2.2, and threshold set at 6000.

RNA extraction

Total RNA was extracted using the Plant RNA Mini Kit (Watson Biotechnologies, Inc., China). RNA aliquots were treated with RNase-free DNase I (TaKaRa, Dalian, China) to remove any DNA contamination. The quality and concentration of total RNA samples were assessed by agarose gel electrophoresis and spectrophotometry.

Sequence analysis and primer design

Primers for amplifying partial sequences of the genes involved in the phenylpropanoid (*PAL* and *C4H*) and flavonoid biosynthesis pathways and of those encoding transcription factors involved in these pathways were designed from conserved nucleotide regions identified by multiple alignments of sequences (Supplementary file 1 at *JXB* online) from *Arabidopsis* and *B. napus* obtained from public databases at the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/BLAST/) and the Arabidopsis Biological Resource Center (http://www.arabidopsis.org/). Gene sequences of *B. rapa* and *B. oleracea* were obtained from public databases at the Brassica database (BRAD; http://brassicadb.org/brad/) and The Brassica oleracea Genome Database (Bolbase; http://www.ocri-genomics.org/bolbase/). Primer sequences used for the qRT-PCR are listed in Supplementary Table S1.

Quantitative real-time PCR

First-strand cDNA was synthesized from 1 μg of each RNA sample in a 20 μl reaction volume with Oligo dT-Adaptor Primer using the RNA PCR Kit (AMV) Ver. 3.0 (TaKaRa). *Brassica napus 26S rRNA* was used as the internal control to monitor sample uniformity of initial RNA input and reverse transcription efficiency. The specific primers were F26S (5'-CACAATGATAGGAAGAGCCGAC-3') and R26S (5'-CAAGGGAACGGGCTTGGCAGAATC-3'). The amplification protocol used for Bn26S was 94 °C for 2 min, followed by 21 cycles of amplification (94 °C for 45 s, 60 °C for 45 s, and 72 °C for 1 min), and then 72 °C for 10 min. Real-time PCR was conducted using SYBR® Premix Ex Taq™ II (Perfect Real Time) (TaKaRa) in a typical 20 μl PCR mixture that included 10 μl of SYBR® Premix Ex Taq™ II, 1–5 μl of template cDNA, and 0.4 μM of each PCR primer. Cycling conditions were 95 °C for 2 min, followed by 40 cycles at 95 °C for 20 s, 56 °C for 1 min, and 72°C for 30 s, and samples were run on the Stratagene Mx3000P Real-Time PCR System (Stratagene) as described previously (Fricker *et al.*, 2007).

Results

Spatiotemporal accumulation of polyphenol compounds in the seed coats of black- and yellow-seeded *B. napus*

To determine the cellular distribution of polyphenol compounds in developing seeds of black- (ZY821) and yellow-seeded (GH06) *B. napus*, a histochemical analysis of seeds harvested at different developmental stages was performed. TBO staining of transverse sections of developing seeds revealed that seed coats lacked polymeric phenolic compounds at 7 DAP (Fig. 1A, H), and that these compounds began to accumulate in the hilum of seeds at 14 DAP (Fig. 1B, I). At these early time points, staining was similar in ZY821 and GH06. At 21 DAP, ZY821 stained more intensely than GH06, indicating that more polymeric phenolic compounds had accumulated in ZY821 (Fig. 1C, J). The distribution of polymeric phenolic compounds was significantly different at

28 DAP. Whereas these compounds were mainly distributed to the palisade and pigment layers of the seed coat in ZY821 (Fig. 1D), they accumulated in the hilum of GH06 seeds (Fig. 1K). At 35 DAP, polymeric phenolic compound accumulation had increased more significantly in the palisade and pigment layers of ZY821 seed coats than in those of GH06 (Fig. 1E, L). However, the pigment layer of GH06 seeds accumulated more polymeric phenolic compounds near the hilum than did that of ZY821 (Fig. 1L). During late developmental stages (42 and 49 DAP), significant differences in polymeric phenolic compounds were particularly obvious in the palisade and pigment layers of the ZY821 and GH06 seed coats (Fig. 1F, G, M, N).

These data show that polymeric phenolic compounds localize mainly to the palisade and pigment layers of the seed coat of black-seeded ZY821. In contrast, these compounds were mainly distributed near the hilum of the pigment layer of yellow-seeded GH06 seed coats (Fig. 1). PA and polyphenol compounds gradually increase during seed maturation and show significant differences in the seed coats of ZY821 and GH06. Moreover, the PA and polyphenol compounds were more abundant in the black-seeded than the yellow-seeded variety of *B. napus* (Fig. 1).

Flavonoid profiling and accumulation in black- and yellow-seeded B. napus

To determine which phenolic compounds accumulated in the seed coats of ZY821 and GH06, seed coats were isolated from seeds of different developmental stages and analysed using LC-UV-MS. Thirty-five phenolic compounds were detected in the flavonoid extracts from both ZY821 and GH06 seed coats. Interestingly, 13 and two compounds were unique to ZY821 and GH06, respectively (Table 1). Three types of flavonol derivatives, including quercetin, isorhamnetin, and kaempferol, and some epicatechin derivatives, were identified. Epicatechin derivatives were only detected in the seed coat extracts of ZY821 (Table 1, Fig. 2).

The concentration of epicatechin, quercetin, isorhametin, and kaempferol derivatives in the coats of ZY821 and GH06 seeds was determined (Fig. 2). It was found that the amount of all four flavonol derivatives increased during seed maturation. The total amount of flavonol derivatives peaked at 42 DAP, and declined thereafter. Epicatechin derivatives, which are reported to be an important type of flavonol in the synthesis of the seed coat of *Arabidopsis* and *Medicago* (Marinova *et al.*, 2007; Zhao and Dixon, 2009), were not detected in the seed coats of GH06 at any stage of seed development.

Additionally, the levels of in-PAs were measured by subjecting the pellet remaining after solvent extraction directly to oxidative cleavage under hot acidic butanol. The seed coat began to accumulate in-PAs at 28 DAP (Fig. 3). A maximum level of 2.0 ± 0.1 μg mg^{-1} of seed coat (fresh weight) was detected during late seed maturation (49 DAP) (Fig. 3). The seed coats of ZY821 exhibited a much higher in-PA content than did those of GH06 (Fig. 3).

Sequence analysis of phenylpropanoid biosynthesis genes from B. napus

The phenylpropanoid biosynthesis pathway is one of the most important secondary metabolism pathways in higher plants. A variety of key phytochemicals, including lignins, stilbenes, coumarins, salicylates, sinapateesters, and flavonoids, are synthesized in this pathway. In *Arabidopsis*, several key genes in this pathway have been cloned and their functions identified (Albert *et al.*, 1997; Nesi *et al.*, 2000, 2001; Baudry *et al.*, 2004). Homologous genes in *B. napus* have also been cloned and characterized to some extent (Chen *et al.*, 2007; Wei *et al.*, 2007; Xu *et al.*, 2007; Lu *et al.*, 2008; Akhov *et al.*, 2009; Auger *et al.*, 2009). To study the expression of flavonoid genes in the seed coats of the ZY821 and GH06

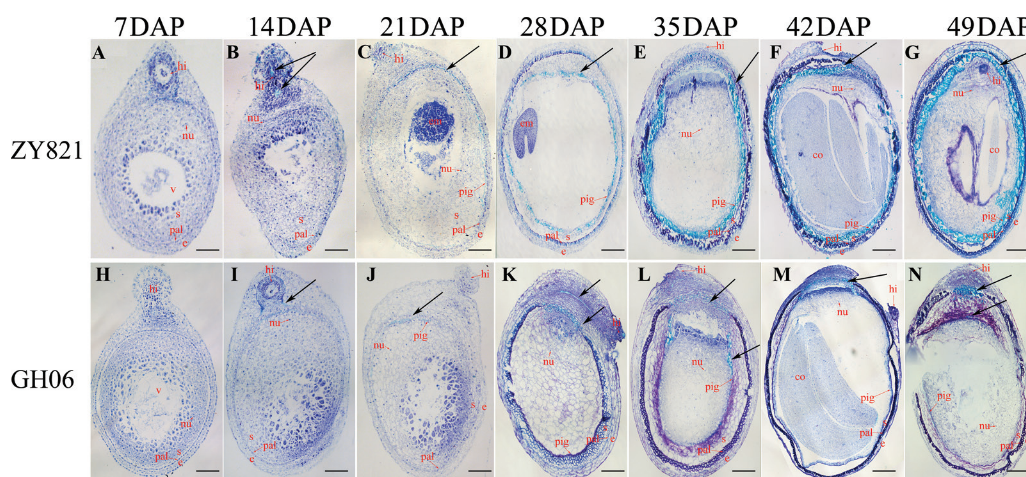

Fig. 1. Changes of proanthocyanidins and phenolic compounds during caryopses development of yellow- and black-seeded *B. napus*. (A–G) Detection and localization of proanthocyanidin and phenolic compounds in seed coat development in ZY821. Bars=20 μm. (H–N) Detection and localization of proanthocyanidin and phenolic compounds in seed coat development in GH06. Bars=20 μm. Black arrows, accumulation site for polymeric phenolic compounds; nu, nucleus; v, vacuole; e, epidermis; s, subepidermis; pal, palisade layer; pig, pigment layer; hi, hilum; em, embryo; co, cotyledon.

Table 1. Main flavonols identified by LC-UV-ESI-MS^2 in seed coat flavonoid extracts from B. napus (ZY821 and GH06).

No.	RT (min)[a]	[M-H][−b]	Putative identity[c]	MS2 [M-H]⁻ (m/z)[b]	UV[d]	ZY821	GH06
1	12.3	463	Quercetin-3-*O*-glucoside	301	64, 324		
2	13.3	386	Putative epicatechin derivative	306, 275, 259, 241, 208	ND		
3	19	447	Kaempferol-3-*O*-glucoside	285^{e}	280, 326		
4	22	787	Quercetin 3-*O*-diglucoside-7-*O*-glucoside	625, 463, 301^{e}	256, 286^{sh}, 358		
5	22.6	423	Putative quercetin derivative	342, 301	266, 342		
6	23.6	771	Kaempferol-3-*O*-diglucoside-7-*O*-glucoside	609, 447, 285	266, 348		
7	24.8	801	Isorhametin-dihexoside-*O*-glucoside	639, 315	254, 284^{sh}, 358		
8	25.4	447	Quercetin-deoxyhex	301		ND	
9	26.9	993	Quercetin 3-*O*-sinapoyldiglucoside-7-*O*-glucoside	831, 625, 301	250, 284^{sh}, 348		
10	28.3	577	[DP2]-B2*	425, 407, 289	222, 264^{sh}, 328		ND
11	28.8	977	Kaempferol-sinapoyl-trihexoside	815, 653, 447	222, 270^{sh}, 330		
12	29.9	561	Unknown	505, 432, 292, 190	ND		
13	30.1	609	Kaempferol-3-*O*-glucoside-7-*O*-glucoside	489, 447, 285	ND		
14	30.5	289	Epicatechin	245, 205, 179^{e}	280		ND
15	30.7	418	Putative quercetin derivative	301	ND	ND	
16	30.7	801	Isorhametin-dihexoside-*O*-glucoside	639, 315	254, 286^{sh}, 354		
17	31.9	477	Isorhametin-3-*O*-glucoside	315	280, 360		
18	32.8	865	[DP3]-	739, 695, 577, 407	280		ND
19	33.9	625	Quercetin-dihexoside	463, 301	280, 316		
20	34.7	639	Isorhametin-dihexoside	477, 315	260, 314		
21	35.5	545	Putative flavonol derivative	432, 292, 190	262, 310		
22	36	609	Sophoraflavonoloside	447,429, 285	ND		
23	36.1	765	Putative quercetin derivative	596, 471,301	284, 368		
24	37.2	557	Isorhametin-hexoside-sulphate	477, 315	ND		
25	37.3	577	[DP2]-B5*	451, 425, 407, 289	270, 338		ND
26	37.9	559	Unknown	432, 292, 190	262, 330		ND
27	38.5	591	Putative quercetin derivative	547, 439, 301	280, 360		ND
28	41.3	495	Putative epicatechin derivative	451, 289	ND		ND
29	41.5	753	Putative epicatechin derivative	529, 457, 429, 289	226, 266, 330		ND
30	44.3	434	Putative epicatechin derivative	289	ND		ND
31	44.8	591	Putative quercetin derivative	547, 439, 395, 301	ND		ND
32	45.6	591	Putative quercetin derivative	559, 439, 367, 301	238, 330		
33	48.4	434	Putative epicatechin derivative	289	ND		ND
34	50.4	418	Putative epicatechin derivative	289	ND		ND
35	54.2	418	Putative epicatechin derivative	289	ND		ND

[a] RT, retention time; measured with a C[18] Grace column and a corresponding gradient profile (see the Materials and methods).
[b] Obtained with an ion trap mass spectrometer.
[c] Compounds were identified after comparison with standards (*) and references.
[d] Obtained with a UV detector.
[e] Standard identified.
ND, none detected; others were detected.

varieties of *B. napus*, expressed sequence tag (EST) sequences of genes involved in this biosynthetic pathway were searched for by blasting the EST sequences of the corresponding genes from *Arabidopsis* against the NCBI database. These gene sequences cloned from ZY821 and GH06 showed a high level of nucleotide similarity with phenylpropanoid and flavonoid genes from other *Brassica* species (Supplementary file 1 at *JXB* online).

The triplication of genes involved in the flavonoid pathway

All current *Brassicaceae* plants are derived from the same postulated *Brassicaceae* ancestry by triplicate genomes. In the *B. rapa* genome, the mesohexaploidy event started between 5 and 9 million years ago (Wang *et al.*, 2011). The *Brassica* mesohexaploidy provides a chance to study gene retention in triplicated genomes. In fact, the substantial gene loss is typical after polyploidy formation in eukaryotes. All copies of TT genes were identified in the *B. rapa* and *B. oleracea* genome (Supplementary Table S2 at *JXB* online) and each of the orthologous blocks corresponding to ancestral blocks was identified using collinearity between orthologues on the genome of *B. rapa* and *B. oleracea* and the *A. thaliana* genome (Table 2). Five copies of C4H were identified and distributed in LF, MF1, MF2, and non-genome triplication in the genome of *B. rapa* and *B. oleracea*, respectively. However, TT7, TT3, TT12, TT8, TTG1, and TT2 were only identified in one copy in the genome of *B. rapa* and *B. oleracea*, respectively. All copies per gene

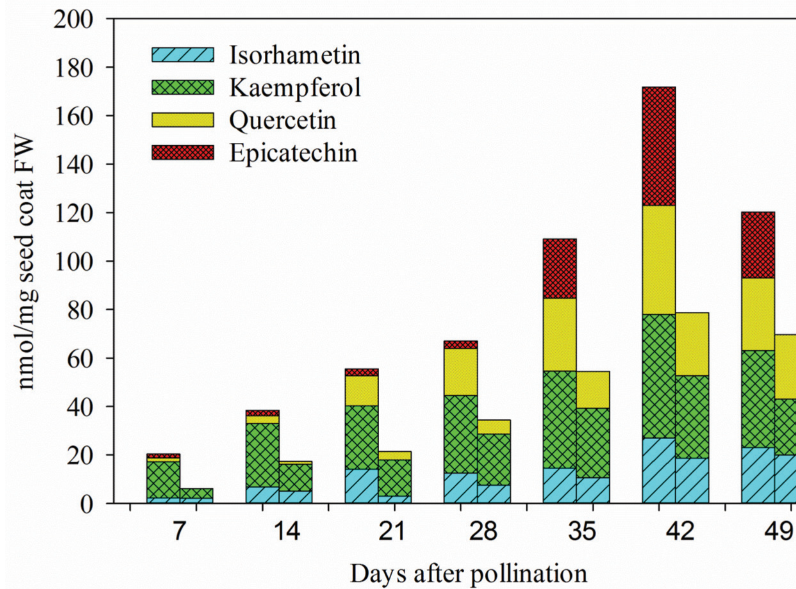

Fig. 2. Flavonoid content in ZY821 and GH06 seed coat during different developmental stages by LC-ESI-MS. Values represent the average of three biological replicates. Isorhamnetin derivates, quercetin derivates, kaempferol derivates, and epicatechin derivates (refer to the colour key depicted on the figure).

were conserved based on alignment of the DNA sequence (Suppplementary file 1).

The expression of genes involved in the biosynthesis of phenolic compounds regulates flavonoid synthesis

BnPAL, which encodes a protein that connects primary and secondary metabolism and transfers phenylalanine to *trans*-cinnamic acid, a precursor of other phenolic compounds (Ohl *et al.*, 1990), is a key gene in phenylpropanoid synthesis in *B. napus*. It was found that the expression level of *BnPAL* was much higher in ZY821 than in GH06, and that expression peaked at 42 and 49 DAP, respectively (Fig. 4A). BnTT4 (BnCHS), which converts three molecules of malonyl-CoA and one molecule of 4-coumaroyl-CoA into nargingenin chalcone, catalyses the first committed step of the flavonoid biosynthesis pathway (Li *et al.*, 1993; Shirley, 1998). All flavonoids and isoflavonoids are derived from the nargingenin chalcone generated by this first enzyme. During both early (7, 14, and 21 DAP) and late (42 and 49 DAP) development stages, *BnTT4* was expressed at the same level in the seed coats of ZY821 and GH06. Only during the middle development stages (28 and 35 DAP) was the expression of *BnTT4* significantly higher in ZY821 than in GH06 (Fig. 4D, E). BnTT5 (BnCHI) stereospecifically directs and greatly accelerates the spontaneous cyclization of chalcones to form the flavonoid core. Since the activity of this enzyme is closely related to BnCHS activity, the gene encoding BnCHS exhibited an expression pattern similar to that of *BnTT4* and *BnTT5*. However, the expression of *BnTT5* peaked after that of *BnTT4* (Fig. 4F). Moreover, *BnTT6*, *BnTT7*, *BnTT3*, and *BnTT18* had expression patterns and levels similar to those of *BnTT4* and *BnTT5*, but they peaked at

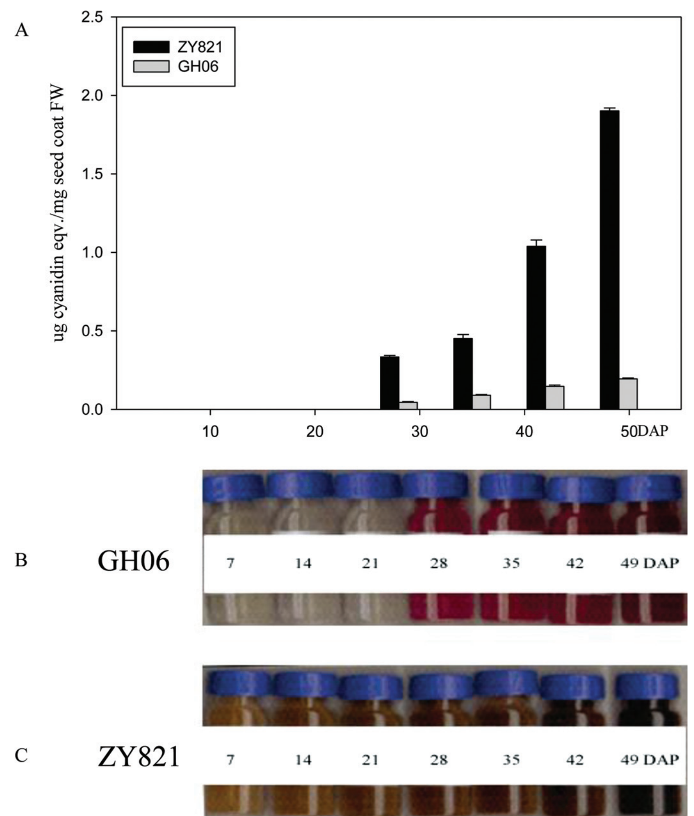

Fig. 3. In-PA content in ZY821 and GH06 *B. napus* seed coats during seed development using BuOH-HCl assays. (A) In-PA content of ZY821and GH06 in the extraction pellet after oxidative cleavage in hot acidic butanol. Values represent the average ±SD of three biological replicates. (B) Extraction of pellets of GH06 using hot acidic butanol. (C) Extraction of pellets of ZY821 using hot acidic butanol.

Table 2. The different copies of TT genes in the B. rapa and B. oleracea genome.

Gene	AGI ID	Triplication block	*B. rapa*					*B. oleracea*				
			LF[a]	MF1[b]	MF2[c]	Non-genome triplication[d]		LF	MF1	MF2	Non-genome triplication	
PAL	AT2G37040	J	Bra005221	Bra017210				Bol025522	Bol037689			
C4H	AT2G30490		Bra018311	Bra021636	Bra022802	Bra022803	Bra021637	Bol006704	Bol004610	Bol033349	Bol033347	Bol004608
TT4	AT5G13930	R	Bra008792	Bra023441	Bra006224	Bra020688	Bra036307	Bol043396	Bol004244	Bol034259		
TT5	AT3G55120	N	Bra007142		Bra003209	Bra007145	Bra017728	Bol044343		Bol008652	Bol018696	Bol044344
TT6	AT3G51240	N	Bra036828		Bra012862			Bol010585		Bol002277		
TT7	AT5G07990	R	Bra009312					Bol043829				
TT3	AT5G42800		Bra027457					Bol035269				
TT18	AT4G22880	U	Bra013652	Bra019350				Bol014986	Bol042059			
TT12	AT3G59030	N			Bra003361					Bol023767		
TT10	AT5G48100	V	Bra037510	Bra020720					Bol033117			
UGT2	AT4G01070	O	Bra037386	Bra000942	Bra008540	Bra008535		Bol011466	Bol010781	Bol040647	Bol040645	
TT8	AT4G09820	P	Bra037887					Bol004077				
TTG1	AT5G24520	Q	Bra009770					Bol022420				
TTG2	AT2G37260	J	Bra005210		Bra023112			Bol025535		Bol005555		
TT2	AT5G35550	S			Bra035532					Bol014029		

[a] LF indicates the least fractionated blocks.
[b] MF1 indicates the medium fractionated blocks.
[c] MF2 indicated the most fractionated blocks.
[d] Non-genome triplication indicates that these copies are not triplicated genome segments.

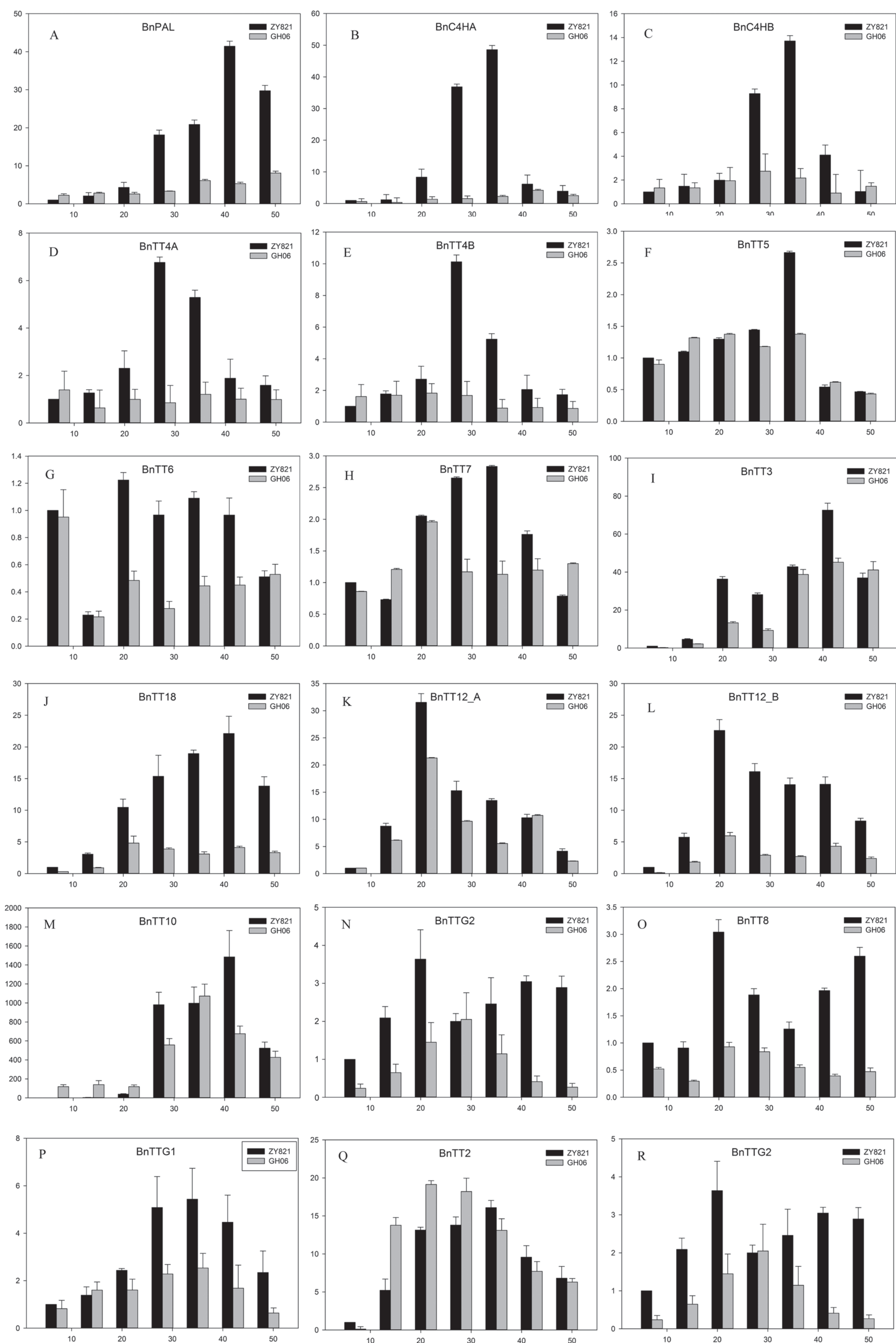

Fig. 4. Analysis of the expression levels of genes involved in flavonoid biosynthesis during seed coat development of ZY821 and GH06 by qRT-PCR (A–O). Seed coat development was divided into seven developmental stages (7, 14, 21, 28, 35, 42, and 49 DAP). Values represent the average ±SD of three biological replicates with three technical replicates of each development stage. Relative gene expression levels were normalized according to the expression values of 7 in ZY821.

different development stages. Whereas *BnTT5*, *BnTT6*, and *BnTT7* expression peaked at 35 DAP (Fig. 4G, H), that of *BnTT3* and *BnTT18* peaked only at 42 DAP (Fig. 4I, J). Furthermore, these genes were expressed at higher levels in ZY821 than in GH06.

In addition, ZY821 and GH06 contain two copies of *BnTT12*, which peak at 28 DAP, and then decline. *BnTT12* was expressed more strongly in ZY821 than in GH06 (Fig. 4K, L). *TT10* encodes an enzyme that oxidizes procyanidins to yield PAs in the Arabidopsis seed coat (Pourcel *et al.*, 2005). In addition to its role in flavonoid biosynthesis, *TT10* also regulates lignin biosynthesis (Liang *et al.*, 2006). The expression of *BnTT10* peaks at 42 DAP (Fig. 4M). However, there was no significant difference in the expression of *BnTT10* between ZY821 and GH06, except at 28 and 42 DAP. *BnUGT2* encodes an epicatechin-specific glucosyltransferase that is involved in the production of epicatechin 3'-*O*-glucoside in the seed coat (Pang *et al.*, 2008). In both ZY821 and GH06 seed coats, *BnUGT2* expression peaked at 35 DAP (Fig. 4N), suggesting that most anthocyanin precursors are synthesized at this stage. The visible function of anthocyanin is to promote colour formation in plant organs (Sparvoli *et al.*, 1994), which supports the hypothesis that anthocyanin accumulation plays an important role in the formation of seed coat colour in *B. napus*

The flavonoid pathway in *Arabidopsis* is known to be regulated not only by structural genes, but also by regulatory genes, such as *TT1*, *TTG1*, *TT2*, *TTG2*, and *TT8* (Walker *et al.*, 1999; Nesi *et al.*, 2000, 2001; Johnson *et al.*, 2002; Sagasser *et al.*, 2002). In this study, *BnTTG1* and *BnTT2* were found to possess a similar expression pattern and showed the highest levels of expression at 35 DAP in the seed coat of ZY821. However, the expression of *BnTT2* peaked sooner in the seed coat of GH06 (Fig. 4P, Q). The expression of *BnTT8* and *BnTTG2* was enhanced at the early stages of seed development (21 and 21 DAP), decreased during the middle stages (35 and 28 DAP), and increased again at the late stages (49 and 42 DAP) (Fig. 4O, R). During most development stages, the expression of *BnTTG1*, *BnTT8*, and *BnTTG2* was stronger in ZY821 than in GH06, and the expression of *BnTT2* was higher in GH06 than in ZY821 at 21 and 28 DAP.

Discussion

Potential mechanisms underlying the histological and biochemical differences in seed coat colour in B. napus

Flavonoids are secondary metabolites that accumulate in vascular plants and, to a lesser extent, in mosses. They promote seed and pollen dispersal by contributing to colour formation in fruits and flowers. Previously, researchers showed that epicatechin derivatives (Marinova *et al.*, 2007; Zhao and Dixon, 2009) and a PA monomer (Holton and Cornish, 1995; Grotewold, 2006) are important flavonols in the synthesis of the seed coat of *Arabidopsis* and *Medicago*.

Recently, much research has focused on deciphering the genetic mechanisms underlying colour formation in *B. napus* seeds, and great progress has been made in this regard. However, there was no consensus on the inheritance model of the seed coat colour trait in *B. napus*. Theander *et al.* (1977) reported that the main factor contributing to seed colour is soluble anthocyanin. Subsequent research showed that the colour of immature seeds is determined by anthocyanidin, polyphenols, and flavonoid, but that the colour of mature seeds is controlled by melanin (Wang and Liu, 1996). PA accumulation contributes to the differences in *B. juncea* (Sharma and Dixon, 2005). In *Arabidopsis*, PAs are synthesized as colourless polymers during the early developmental stages, and are then oxidized into brown complexes that cross-link within the cell during seed maturation. These modifications account for the typical brown colour of the *Arabidopsis* wild-type testa (Stafford, 1988; Kitamura *et al.*, 2004; Pourcel *et al.*, 2005). In *B. napus*, PAs accumulated strongly in the black-seeded line, but weakly in the yellow-seeded line. The flavonol content, however, did not differ much between these two lines (Akhov *et al.*, 2009). In agreement with these findings, Marles and Gruber (2004) found that PA was deposited over the entire seed coat of the dark-seeded germplasm and in patches in some yellow-seeded germplasms. However, the correlation between total flavonoid content and seed colour was comparatively weak (Lipsa *et al.*, 2007).

In this study, it was shown that flavonoid and its derivatives, including epicatechin, quercetin, isorhametin, and kaempferol, mainly accumulated in the black-seeded variety. In addition, 35 phenolic compounds were detected in the flavonoid extracts from both ZY821 and GH06 seed coats according to the retention time analysis of LC-UV-MS. Interestingly, 13 and two compounds could be detected only in ZY821 and GH06, respectively (Table 1). Moreover, epicatechin derivatives were not detected in the seed coat extracts of GH06 (Table 1, Fig. 2). Large differences were also found in the polyphenol composition of seed coats from the black- and yellow-seeded lines. The cytological analysis showed that polymeric phenol compounds began to accumulate in the hilum of seeds during the early developmental stages, and were mainly confined to the palisade and pigment layers of the seed coat during the middle to late stages of development. Moreover, PA and polyphenol content were higher in ZY821 than in GH06 seed coats (Fig. 1). In addition, the in-PAs were not detected during the early developmental stages of *B. napus*, but accumulated during the middle to late stages, and the in-PA content of the black-seeded variety was much higher than that of the yellow-seeded line. It was also found that in-PAs accumulated in the seed coats of GH06, but did not vary much during development (Fig. 3A). The differences in in-PA content may be due to differences in the synthesis of epicatechin, as epicatechin is thought to be the major precursor of in-PAs in *Arabidopsis* seed coats (Lepiniec *et al.*, 2006). These results suggest that the differences in seed coat colour observed in *B. napus* are largely attributable to differences in the content of epicatechin and its derivatives and of in-PAs.

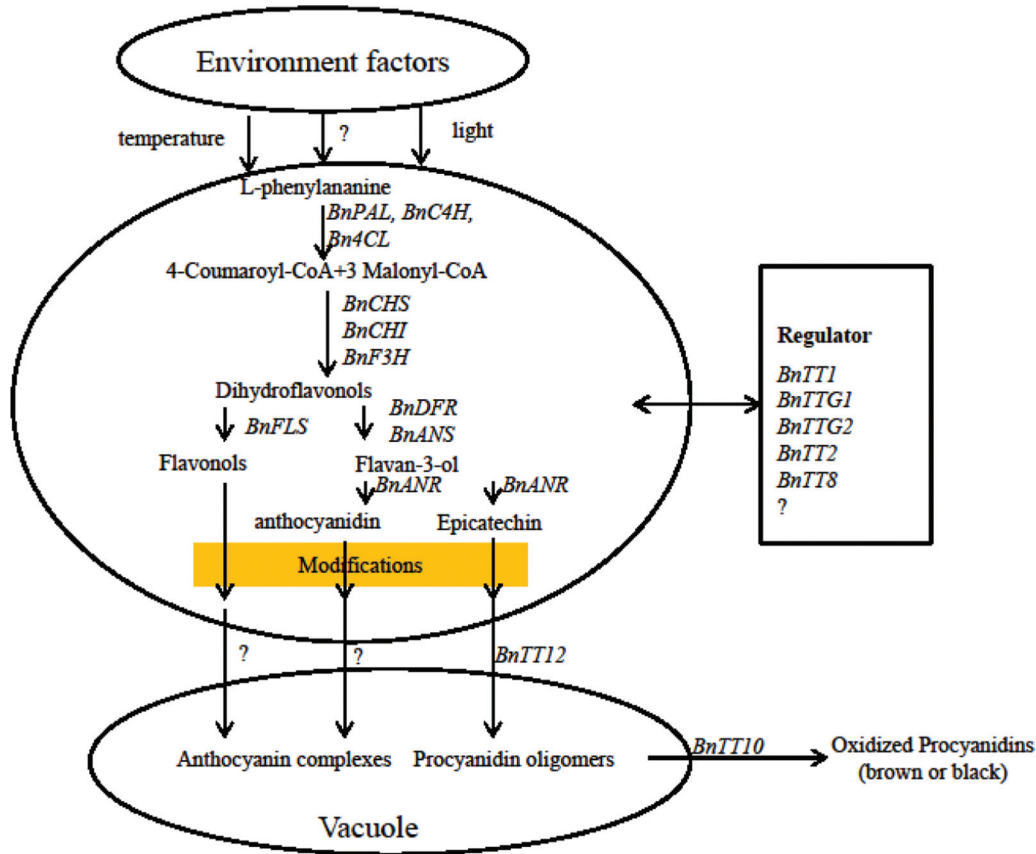

Fig. 5. Proposed model for the molecular mechanisms underlying seed coat colour in *B. napus*. *BnPAL*, L-phenylalanine ammonia-lyase; *BnC4H*, cinnamate 4-hydroxylase; *Bn4CL*, 4-coumarate: CoA ligase; *BnCHS*, chalcone synthase; *BnCHI*, chalcone isomerase; *BnF3H*, flavanone 3-hydroxylase; *BnDFR*, dihydroflavonol reductase; *BnANS*, anthocyanidin synthase; *BnANR*, anthocyanidin reductase.

The expression of genes involved in the flavonoid biosynthesis pathway in the seed coats of black- and yellow-seeded B. napus

Several key genes involved in flavonoid biosynthesis have been cloned and their functions identified by analysing various mutants in *Arabidopsis* (Albert *et al.*, 1997; Nesi *et al.*, 2000, 2001; Baudry *et al.*, 2004; Arsovski *et al.*, 2010; Le *et al.*, 2010) and other species (Pang *et al.*, 2004, 2008; Gallardo *et al.*, 2007; Jiang and Deyholos, 2010). Homologous genes in the *B. napus* pathway have also been cloned and characterized (Chen *et al.*, 2007; Wei *et al.*, 2007; Xu *et al.*, 2007; Lu *et al.*, 2008; Akhov *et al.*, 2009; Auger *et al.*, 2009). In *B. napus*, BnPAL and BnC4H are the key enzymes of the phenylpropanoid pathway, which synthesizes primary and secondary metabolites and converts phenylalanine into *trans*-cinnamic acid, which is a precursor of other phenolic compounds (Ohl *et al.*, 1990). Blount and co-workers reported that PAL activity was reduced by the antisense-mediated silencing of *C4H*, and concluded that *C4H* was involved in regulating the expression of *PAL* (Blount *et al.*, 2000). In a previous study, using three pairs of near isogenic lines of *B. napus*, it was found that the enzyme activity of BnPAL was much higher in the black-seeded lines than in the yellow-seeded lines, and differed significantly among the lines. Moreover, it was found that cinnamic acid accumulated robustly and *p*-coumaric acid weakly in the young seed coats of yellow-seeded lines, but conversely during the middle to late stages of seed development (Liang and Li, 2004).

In this study, the expression level of *BnPAL* in ZY821 was found to be much higher than in GH06, and expression in ZY821 was strongest at 42 DAP, which was also 7 d earlier than in GH06 (Fig. 4A). These results are consistent with the PAL activity and expression in *B. napus*, and confirm that the activity of BnPAL is closely related to the expression pattern of *BnPAL*. Furthermore, the expression level of *BnC4H* was much higher in ZY821 than in GH06 during the middle developmental stages, and the expression of *BnC4H* peaked 7 d before that of *BnPAL* (Fig. 4A–C). This result supports the hypothesis that flux into the phenylpropanoid pathway is controlled, at least in part, by feedback regulation of PAL sensed through the production of cinnamic acid (Blount *et al.*, 2000). In addition, it also confirmed the previous results that showed that cinnamic acid accumulated robustly and *p*-coumaric acid weakly in the young seed coats of yellow-seeded lines, but conversely during the middle to late stages of seed development (Liang and Li, 2004).

UGT2 encodes an epicatechin-specific glucosyltransferase involved in the production of epicatechin 3'-*O*-glucoside in the seed coat (Pang *et al.*, 2008). *TT12*

is an ABC transporter that is responsible for epicatechin 3'-*O*-glucoside and anthocyanin cyanidin-3-*O*-glucoside uptake in *Medicago* and *Arabidopsis*, respectively (Debeaujon *et al.*, 2001; Marinova *et al.*, 2007; Chai *et al.*, 2009; Zhao and Dixon, 2009). Moreover, ANR converts cyanidin into epicatechin. Epicatechin is one of the precursors for biosynthesis of PA (Albert *et al.*, 1997; Devic *et al.*, 1999; Xie *et al.*, 2003; Baudry *et al.*, 2004). In this study, the expression of *BnUGT2* and *BnTT12* was found to be higher in ZY821 than in GH06; however, both genes were expressed throughout the development of GH06 seed coats (Fig. 4N, K, L). This result also indicates that epicatechin, which was not detected at any stage of seed coat development in GH06, contributes to seed coat colour formation (Fig. 2).

It was shown here that the expression of genes involved in flavonoid biosynthesis varied throughout seed development, and that the genes had different expression patterns in the black- and yellow-seeded lines. The genes were classified according to the time at which their expression peaked. The expression of the first group of genes, including *BnTT4*, *BnTT8*, and *BnTT12*, peaked at the early stages of seed development in black-seeded ZY821. Another group of genes (*BnC4H*, *BnTT5*, *BnTT6*, *BnTT7*, *BnUGT2 BnPAL*, *BnTT3*, *BnTT18*, and *BnTT10*) showed the highest expression levels at 35 and 42 DAP, respectively. Thus, these genes could play important roles in flavonoid biosynthesis during the middle to late stages of seed coat development (Fig. 4). In addition, *BnTT6*, *BnTT7*, *BnTT3*, and *BnTT18* exhibited similar expression patterns between the black- and yellow-seeded varieties of *B. napus*. In contrast, the expression of *BnTT7*, *BnTT18*, and *BnTT10* peaked earlier in GH06 than in ZY821 (Fig. 4H, J, M). Furthermore, there were no significant differences in the expression level of *BnPAL*, *BnC4H*, *BnTT4*, and *BnTT6* between the different stages of seed development in GH06. The differences in the expression patterns of genes revealed genetic differences in the synthesis of pigments between the black- and yellow-seeded varieties of *B. napus*. These results suggested that *BnPAL*, *BnC4H*, *BnTT4*, and *BnTT6* are the key genes underlying differences in seed coat pigmentation in *B. napus*, and that the expression of these genes may be inhibited in the yellow-seeded line of *B. napus*.

Regulation mechanisms underlying the biosynthesis of seed coat pigment in B. napus

The inheritance of seed coat colour in *B. napus* is quite complicated, and the regulatory mechanisms underlying the biosynthesis of the relevant genes were hitherto unknown. In this study, the expression level of genes involved in pigment biosynthesis was found to vary greatly between the yellow- and black-seeded lines of *B. napus*. However, all of these genes were also expressed in the yellow-seeded lines. These data indicate that the absence of pigment synthesis in the yellow-seeded line of *B. napus* involves the down-regulation, but not the complete inactivation, of several key genes. In addition, the seed coat colour of *B. napus* was affected by environmental factors, such as temperature and light (Deynze *et al.*, 1993). Future research should aim to decipher the mechanisms that down-regulate the expression of genes involved in pigment synthesis in yellow-seeded *B. napus*.

For the reasons mentioned above, this study has almost certainly analysed the metabolic networks underlying pigment formation, investigated the laws of variation in the accumulation of flavonol and phenolic compounds using histochemical and LC-UV-MS methods, and monitored the differential expression of the main structural genes and transcription factors involved in the flavonoid biosynthesis pathway (Fig. 5). The findings illuminate the mechanisms underlying colour formation in *B. napus* seed coats.

Supplementary data

Supplementary data are available at *JXB* online.

Figure S1. The pathway of flavonoid biosynthesis, modification, and transport in *Arabidopsis*.

Figure S2. The phenotype of GH06 and ZY821.

Table S1. Specific primers used for qRT-PCR.

Table S2. The distribution of TT genes in the genome of *B. rapa* and *B. oleracea.*

Supplementary file 1. Multiple alignments of sequences obtained from the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/BLAST/) and the Arabidopsis Biological Resource Center (http://www.arabidopsis.org/) database.

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References

Abrahams S, Lee E, Walker AR, Tanner GJ, Larkin PJ, Ashton AR. 2003. The Arabidopsis TDS4 gene encodes leucoanthocyanidin dioxygenase (LDOX) and is essential for proanthocyanidin synthesis and vacuole development. *The Plant Journal* **35,** 624–636.

Akhov LAL, Ashe PAP, Tan YTY, Datla RDR, Selvaraj GSG. 2009. 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. *Botany* **87,** 616–625.

Albert S, Delseny M, Devic M. 1997. *BANYULS*, a novel negative regulator of flavonoid biosynthesis in the *Arabidopsis* seed coat. *The Plant Journal* **11,** 289–299.

Arsovski AA, Haughn GW, Western TL. 2010. Seed coat mucilage cells of Arabidopsis thaliana as a model for plant cell wall research. *Plant Signaling and Behavior* **5,** 796.

Auger B, Baron C, Lucas MO, Vautrin S, Bergès H, Chalhoub B, Fautrel A, Renard M, Nesi N. 2009. *Brassica* orthologs from *BANYULS* belong to a small multigene family, which is involved in procyanidin accumulation in the seed. *Planta* **230,** 1167–1183.

Auger, B, Marnet N, Gautier V, Maia-Grondard A, Leprince F, Renard M, Guyot S, Nesi N, Routaboul JM. 2010. A detailed survey of seed coat flavonoids in developing seeds of *Brassica napus* L. *Journal of Agricultural and Food Chemistry* **58,** 6246–6256.

Baudry A, Heim MA, Dubreucq B, Caboche M, Weisshaar B, Lepiniec L. 2004. TT2, TT8, and TTG1 synergistically specify the expression of BANYULS and proanthocyanidin biosynthesis in *Arabidopsis thaliana*. *The Plant Journal* **39,** 366–380.

Bharti AK, Khurana JP. 2003. Molecular characterization of transparent testa (tt) mutants of *Arabidopsis thaliana* (ecotype Estland) impaired in flavonoid biosynthetic pathway. *Plant Science* **165,** 1321–1332.

Blount JW, Korth KL, Masoud SA, Rasmussen S, Lamb C, Dixon RA. 2000. Altering expression of cinnamic acid 4-hydroxylase in transgenic plants provides evidence for a feedback loop at the entry point into the phenylpropanoid pathway. *Plant Physiology* **122,** 107–116.

Burbulis IE, Winkel-Shirley B. 1999. Interactions among enzymes of the *Arabidopsis flavonoid* biosynthetic pathway. *Proceedings of the National Academy of Sciences, USA* **96,** 12929–12934.

Chai YR, Lei B, Huang HL, Li JN, Yin JM, Tang ZL, Wang R, Chen L. 2009. *TRANSPARENT TESTA*12 genes from *Brassica napus* and parental species: cloning, evolution, and differential involvement in yellow seed trait. *Molecular Genetics and Genomics* **281,** 109–123.

Chen AH, Chai YR, Li JN, Chen L. 2007. Molecular cloning of two genes encoding cinnamate 4-hydroxylase (C4H) from oilseed rape (*Brassica napus*). *Journal of Biochemistry and Molecular Biology* **40,** 247–260.

Chiu LW, Zhou X, Burke S, Wu X, Prior RL, Li L. 2010. The purple cauliflower arises from activation of a MYB transcription factor. *Plant Physiology* **154,** 1470–1480.

Debeaujon I, Peeters AJ M, Léon-Kloosterziel KM, Koornneef M. 2001. The *TRANSPARENT TESTA12* gene of *Arabidopsis* encodes a multidrug secondary transporter-like protein required for flavonoid sequestration in vacuoles of the seed coat endothelium. *The Plant Cell* **13,** 853.

Devic M, Guilleminot J, Debeaujon I, Bechtold N, Bensaude E, Koornneef M, Pelletier G, Delseny M. 1999. The *BANYULS* gene encodes a DFR-like protein and is a marker of early seed coat development. *The Plant Journal* **19,** 387–398.

Deynze AEV, Beversdorf WD, Pauls KP. 1993. Temperature effects on seed color in black- and yellow-seeded rapeseed. *Canadian Journal of Plant Science* **73,** 383–387.

Fricker M, Messelhausser U, Busch U, Scherer S, Ehling-Schulz M. 2007. Diagnostic real-time PCR assays for the detection of emetic Bacillus cereus strains in foods and recent food-borne outbreaks. *Applied and Environmental Microbiology* **73,** 1892.

Fu F-Y, Lie-Zhao L, Chai Y-R, Chen L, Yang T, Jin M-Y, Ma A-F, Yan X-Y, Zhang Z-S. 2007. Localization of QTLs for seed color using recombinant inbred lines of *Brassica napus* in different environments. *Genome* **50,** 840–854.

Gachon C, Langlois-Meurinne M, Henry Y, Saindrenan P. 2005. Transcriptional co-regulation of secondary metabolism enzymes in *Arabidopsis*: functional and evolutionary implications. *Plant Molecular Biology* **58,** 229–245.

Gallardo K, Firnhaber C, Zuber H, Héricher D, Belghazi M, Henry C, Küster H, Thompson R. 2007. A combined proteome and transcriptome analysis of developing *Medicago truncatula* seeds. *Molecular and Cellular Proteomics* **6,** 2165–2179.

Grotewold E. 2006. The genetics and biochemistry of floral pigments. *Annual Review of Plant Biology* **57,** 761–780.

Hoffmann T, Kalinowski G, Schwab W. 2006. RNAi-induced silencing of gene expression in strawberry fruit (*Fragaria×ananassa*) by agroinfiltration: a rapid assay for gene function analysis. *The Plant Journal* **48,** 818–826.

Holton TA, Cornish EC. 1995. Genetics and biochemistry of anthocyanin biosynthesis. *The Plant Cell* **7,** 1071–1083.

Jiang Y, Deyholos MK. 2010. Transcriptome analysis of secondary-wall-enriched seed coat tissues of canola (*Brassica napus* L.). *Plant Cell Reports* **29,** 327–342.

Johnson CS, Kolevski B., Smyth DR. 2002. TRANSPARENT TESTA GLABRA2, a trichome and seed coat development gene of Arabidopsis, encodes a WRKY transcription factor. *The Plant Cell* **14,** 1359–1375.

Kasai A, Kasai K, Yumoto S, Senda, M. 2007. Structural features of GmIRCHS, candidate of the I gene inhibiting seed coat pigmentation in soybean: implications for inducing endogenous RNA silencing of chalcone synthase genes. *Plant Molecular Biology* **64,** 467–479.

Kitamura S, Shikazono N, Tanaka A. 2004. *TRANSPARENT TESTA* 19 is involved in the accumulation of both anthocyanins and proanthocyanidins in *Arabidopsis*. *The Plant Journal* **37,** 104–114.

Le BH, Cheng C, Bui AQ, Wagmaister JA, Henry KF, Pelletier J, Kwong L, Belmont M, Kirkbride R, Horvath S. 2010. Global analysis of gene activity during *Arabidopsis* seed development and identification of seed-specific transcription factors. *Proceedings of the National Academy of Sciences, USA* **107,** 8063–8070.

Lepiniec L, Debeaujon I, Routaboul JM, Baudry A, Pourcel L, Nesi N, Caboche M. 2006. Genetics and biochemistry of seed flavonoids. *Annual Review of Plant Biology* **57,** 405–430.

Li J, Ou-Lee TM, Raba R, Amundson RG, Last RL. 1993. *Arabidopsis* flavonoid mutants are hypersensitive to UV-B irradiation. *The Plant Cell* **5,** 171–179.

Liang M, Davis E, Gardner D, Cai X, Wu Y. 2006. Involvement of AtLAC15 in lignin synthesis in seeds and in root elongation of Arabidopsis. *Planta* **224,** 1185–1196.

Liang Y, Li JN. 2004. Relationship of the color formation and related enzymes and protein content of the spermoderms in *Brassica napus*. *Scientia Agricultura Sinica* **37,** 522–527.

Lin LZ, Harnly JM. 2009. Identification of the phenolic components of collard greens, kale, and Chinese broccoli. *Journal of Agricultural and Food Chemistry* **57,** 7401–7408.

Lin LZ, Harnly JM. 2010. Phenolic component profiles of mustard greens, yu choy, and 15 other *Brassica* vegetables. *Journal of Agricultural and Food Chemistry* **58,** 6850–6857.

Lipsa F, Snowdon R, Friedt W. 2007. Improving rapeseed meal quality by reduction of condensed tannins. *Proceedings of the 12th International Rapeseed Congress*, Wuhan, China, 135–137.

Liu Z-w, Ting-dong F, Jin-xing T, Bao-yuan C. 2005. Inheritance of seed colour and identification of RAPD and AFLP markers linked to the seed colour gene in rapeseed (*Brassica napus* L.). *Theoretical and Applied Genetics* **110,** 303–310.

Lu K, Chai YR, Zhang K, Wang R, Chen L, Lei B, Lu J, Xu XF, Li JN. 2008. Cloning and characterization of phosphorus starvation inducible *Brassica napus PURPLE ACID PHOSPHATASE*12 gene family, and imprinting of a recently evolved MITE-minisatellite twin structure. *Theoretical and Applied Genetics* **117,** 963–975.

Marinova K, Pourcel L, Weder B, Schwarz M, Barron D, Routaboul JM, Debeaujon I, Klein M. 2007. The *Arabidopsis* MATE transporter TT12 acts as a vacuolar flavonoid/H+-antiporter active in proanthocyanidin-accumulating cells of the seed coat. *The Plant Cell* **19,** 2023–2038.

Marles M, Gruber MY. 2004. Histochemical characterisation of unextractable seed coat pigments and quantification of extractable lignin in the *Brassicaceae*. *Journal of the Science of Food and Agriculture* **84,** 251–262.

Meng J, Shi S, Gan L, Li Z, Qu X. 1998. The production of yellow-seeded *Brassica napus* (AACC) through crossing interspecific hybrids of *B. campestris* (AA) and *B. carinata* (BBCC) with *B. napus*. *Euphytica* **103,** 329–333.

Nesi N, Debeaujon I, Jond C, Pelletier G, Caboche M, Lepiniec L. 2000. The *TT8* gene encodes a basic helix–loop–helix domain protein required for expression of DFR and BAN genes in *Arabidopsis* siliques. *The Plant Cell* **12,** 1863–1878.

Nesi N, Jond C, Debeaujon I, Caboche M, Lepiniec L. 2001. The *Arabidopsis TT2* gene encodes an R2R3 MYB domain protein that acts as a key determinant for proanthocyanidin accumulation in developing seed. *The Plant Cell* **13,** 2099–2114.

Nesi N, Lucas MO, Auger B, Baron C, Lécureuil A, Guerche P, Kronenberger J, Lepiniec L, Debeaujon I, Renard M. 2009. The promoter of the *Arabidopsis thaliana BAN* gene is active in proanthocyanidin-accumulating cells of the *Brassica napus* seed coat. *Plant Cell Reports* **28,** 601–617.

Ohl S, Hedrick SA, Chory J, Lamb CJ. 1990. Functional properties of a phenylalanine ammonia-lyase promoter from *Arabidopsis*. *The Plant Cell* **2,** 837–848.

Olsson G. 1960. Species crosses within the genus *Brassica*. I. Artificial *Brassica juncea* Coss. *Hereditas* **46,** 171–223.

Pang JS, He MY, Liu B. 2004. Construction of the seed-coat cDNA microarray and screening of differentially expressed genes in barley. *Acta Biochimica et Biophysica Sinica* **36,** 695–700.

Pang Y, Peel GJ, Sharma SB, Tang Y, Dixon RA. 2008. A transcript profiling approach reveals an epicatechin-specific glucosyltransferase expressed in the seed coat of Medicago truncatula. *Proceedings of the National Academy of Sciences, USA* **105,** 14210–14215.

Pelletier MK, Shirley BW. 1996. Analysis of flavanone 3-hydroxylase in *Arabidopsis* seedlings (Coordinate regulation with chalcone synthase and chalcone isomerase). *Plant Physiology* **111,** 339–345.

Pourcel L, Routaboul JM, Kerhoas L, Caboche M, Lepiniec L, Debeaujon I. 2005. *TRANSPARENT TESTA10* encodes a laccase-like enzyme involved in oxidative polymerization of flavonoids in *Arabidopsis* seed coat. *The Plant Cell* **17,** 2966–2980.

Romani A, Vignolini P, Isolani L, Ieri F, Heimler D. 2006. HPLC-DAD/MS characterization of flavonoids and hydroxycinnamic derivatives in turnip tops (*Brassica rapa* L. subsp. sylvestris L.). *Journal of Agricultural and Food Chemistry* **54,** 1342–1346.

Russell DW. 1971. The metabolism of aromatic compounds in higher plants. *Journal of Biological Chemistry* **246,** 3870–3878.

Sagasser M, Lu GH, Hahlbrock K, Weisshaar B. 2002. A. thaliana TRANSPARENT TESTA 1 is involved in seed coat development and defines the WIP subfamily of plant zinc finger proteins. *Genes and Development* **16,** 138–149.

Schoenbohm C, Martens S, Eder C, Forkmann G, Weisshaar B. 2000. Identification of the *Arabidopsis thaliana* flavonoid 3'-hydroxylase gene and functional expression of the encoded P450 enzyme. *Biological Chemistry* **381,** 749–753.

Sharma SB, Dixon RA. 2005. Metabolic engineering of proanthocyanidins by ectopic expression of transcription factors in *Arabidopsis thaliana*. *The Plant Journal* **44,** 62–75.

Shirley BW. 1998. Flavonoids in seeds and grains: physiological function, agronomic importance and the genetics of biosynthesis. *Seed Science Research* **8,** 415–422.

Somers DJ, Rakow G, Prabhu VK, Friesen KR. 2001. Identification of a major gene and RAPD markers for yellow seed coat colour in *Brassica napus*. *Genome* **44,** 1077–1082.

Sparvoli F, Martin C, Scienza A, Gavazzi G, Tonelli C. 1994. Cloning and molecular analysis of structural genes involved in flavonoid and stilbene biosynthesis in grape (Vitis vinifera L.). *Plant Molecular Biology* **24,** 743–755.

Stafford HA. 1988. Proanthocyanidins and the lignin connection. *Phytochemistry* **27,** 1–6.

Stringam G, McGregor D, Pawlowski S. 1974. Chemical and morphological characteristics associated with seed coat color in rapeseed. In: *Proceedings of the 4th International Rapeseed Congress*, Giessen, Germany, June 4–8, 1974.

Sun Y, Li H, Huang JR. 2012. Arabidopsis TT19 functions as a carrier to transport anthocyanin from the cytosol to tonoplasts. *Molecular Plant* **5,** 387–400.

Tang Z, Li J, Zhang X, Chen L, Wang R. 1997. Genetic variation of yellow-seeded rapeseed lines (*Brassica napus* L.) from different genetic sources. *Plant Breeding* **116,** 471–474.

Theander O, Aman P, Miksche GE, Yasuda S. 1977. Carbohydrate, polyphenols, and lignin in seed hulls of different colours from tumip rapeseed. *Journal of Agricultural and Food Chemistry* **25,** 270–273.

Walker AR, Davison PA, Bolognesi-Winfield AC, James CM, Srinivasan N, Blundell TL, Esch JJ, Marks MD, Gray JC. 1999. The TRANSPARENT TESTA GLABRA1 locus, which regulates trichome differentiation and anthocyanin biosynthesis in Arabidopsis, encodes a WD40 repeat protein. *The Plant Cell* **11,** 1337–1350.

Wang H, Liu HL. 1996. Quantitative variation of anthocyanidins, polyphenols, trans-cinnamic acid and PAL activity in seed hulls of black- and yellow-seeded *B. napus*. *Journal of Huazhong Agricultural University* **15,** 509–513.

Wang X, Wang H, Wang J, *et al.* 2011. The genome of the mesopolyploid crop species Brassica rapa. *Nature Genetics* **43,** 1035–1039.

Wei YL, Li JN, Lu J, Tang ZL, Pu DC, Chai YR. 2007. Molecular cloning of *Brassica napus TRANSPARENT TESTA 2* gene family encoding potential MYB regulatory proteins of proanthocyanidin biosynthesis. *Molecular Biology Reports* **34,** 105–120.

Winkel-Shirley B. 2001. Flavonoid biosynthesis. A colorful model for genetics, biochemistry, cell biology, and biotechnology. *Plant Physiology* **126,** 485.

Winkel-Shirley B. 2002. Biosynthesis of flavonoids and effects of stress. *Current Opinion in Plant Biology* **5,** 218–223.

Wisman E, Cardon GH, Fransz P, Saedler H. 1998. The behaviour of the autonomous maize transposable element En/Spm in *Arabidopsis thaliana* allows efficient mutagenesis. *Plant Molecular Biology* **37,** 989–999.

Xiao SS, Xu JS, Li Y, Zhang L, Shi S, Shi S, Wu JS, Liu KD. 2007. Generation and mapping of SCAR and CAPS markers linked to the seed coat color gene in *Brassica napus* using a genome-walking technique. *Genome* **50,** 611–618.

Xie DY, Sharma SB, Paiva NL, Ferreira D, Dixon RA. 2003. Role of anthocyanidin reductase, encoded by *BANYULS* in plant flavonoid biosynthesis. *Science* **299,** 396.

Xu BB, Li JN, Zhang XK, Wang R, Xie LL, Chai YR. 2007. Cloning and molecular characterization of a functional flavonoid 3'-hydroxylase gene from *Brassica napus*. *Journal of Plant Physiology* **164,** 350–363.

Zhang Y, Li X, Chen W, Yi B, Wen J, Shen J, Ma C, Chen B, Tu J, Fu T. 2011. Identification of two major QTL for yellow seed color in two crosses of resynthesized *Brassica napus* line No. 2127-17. *Molecular Breeding* **28,** 335–342.

Zhao J, Dixon RA. 2009. MATE transporters facilitate vacuolar uptake of epicatechin 3'-O-glucoside for proanthocyanidin biosynthesis in *Medicago truncatula* and *Arabidopsis*. *The Plant Cell* **21,** 2323–2340.

Zhao J, Pang Y, Dixon RA. 2010. The mysteries of proanthocyanidin transport and polymerization. *Plant Physiology* **153,** 437–443.