# **RESEARCH ARTICLE**

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# Transcriptomic comparison between developing seeds of yellow- and blackseeded *Brassica napus* reveals that genes influence seed quality



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# Abstract

**Background:** *Brassica napus* is of substantial economic value for vegetable oil, biofuel, and animal fodder production. The breeding of yellow-seeded *B. napus* to improve seed quality with higher oil content, improved oil and meal quality with fewer antinutrients merits attention. Screening the genes related to this phenotype is valuable for future rapeseed breeding.

**Results:** A total of 85,407 genes, including 4317 novel genes, were identified in the developing seeds of yellow- and black-seeded *B. napus*, and yellow rapeseed was shown to be an introgression line between black-seeded *B. napus* and yellow-seeded *Sinapis alba*. A total of 15,251 differentially expressed genes (DEGs) were identified among all the libraries, and 563 and 397 common DEGs were identified throughout black and yellow seed development, including 80 upregulated and 151 downregulated genes related to seed development and fatty acid accumulation. In addition, 11 up-DEGs and 31 down-DEGs were identified in all developmental stages of yellow rapeseed compared with black seed. Enrichment analysis revealed that many DEGs were involved in biosynthetic processes, pigment metabolism, and oxidation-reduction processes, such as flavonoid and phenylpropanoid biosynthesis, phenylalanine metabolism, flavone and flavonoi biosynthesis, and fatty acid biosynthesis and metabolism. We found that more than 77 DEGs were related to flavonoid and lignin biosynthesis, including *4CL*, *C4H*, and *PAL*, which participated in phenylalanine metabolism, and *BAN*, *CHI/TT5*, *DFR*, *F3H*, *FLS*, *LDOX*, *PAP*, *CHS/TT4*, *TT5*, *bHLH/TT8*, *WD40*, *MYB*, *TCP*, and *CYP*, which were involved in flavonoid biosynthesis. Most of these DEGs were downregulated in yellow rapeseed and were consistent with the decreased flavonoid and lignin contents. Both up- and down-DEGs related to fatty acid biosynthesis and metabolism were also analyzed, which could help to explain the improved oil content of yellow rapeseed.

**Conclusion:** This research provided comprehensive transcriptome data for yellow-seeded *B. napus* with a unique genetic background, and all the DEGs in comparison with the black-seeded counterpart could help to explain seed quality differences, such as lower pigmentation and lignin contents, and higher oil content.

Keywords: Brassica napus, Seed coat color, Flavonoid biosynthesis, Fatty acid, Gene expression

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## Background

Brassica napus, as the third leading oilseed crop grown worldwide, greatly contributes to providing edible oils, biofuels, and animal fodder [1]. Rapeseed with increased oil content, better oil and meal quality, and improved yield has been the main breeding drive during the past decades [2]. Yellow-seeded B. napus was improved with better seed oil and meal quality due to fewer pigments and polyphenols; thus, breeding yellow rapeseed has been preferred for decades, and studies on the molecular mechanism of this phenotype have been reported as well [3]. Fewer antinutrients, including phenolic compounds, tannins, proanthocyanins (PAs), and lignin, in yellow rapeseed were correlated with the flavonoid biosynthetic, phenylpropanoid, and phenylalanine/tyrosine metabolic pathways with common substrates, such as coumaroyl CoA, caffeoyl CoA, and feruloyl CoA [4-6]. Hitherto, yellow-seeded B. napus has been selected from interspecific hybridization of Brassica species and intergeneric hybridization [7, 8]. As reported in Arabidopsis thaliana, mutations in transparent testa (TT) genes are responsible for seed coat color variations, including early (EBGs) and late (LBGs) biosynthesis genes [9]. The EBGs include TT4/CHS, TT5/CHI, TT6/F3H, and TT7/F3'H, and LBGs include TT3/DFR, TT18/LDOX/ANS, BAN/ ANR, TT12, TT19/GSTF12/GST26, and AHA10. In addition, the MYB-bHLH-WD40 (MBW) complex has been reported to have important regulatory functions in anthocyanin accumulation. Four MBW have been reported, including TT2-TT8-TTG1, MYB5-TT8-TTG1, TT2-EGL3-TTG1, and TT2-GL3-TTG1 [10]. Appelhagen et al. (2011) found that PAP1/MYB75 and PAP2/ MYB90 participated in the formation of MBW [11]. Homologous TTs related to seed coat variation in Brassicaceae have been delineated, including 95 copies of 21 TTs in B. napus [3]. Previously, F3'H, TT2, PAL, BAN, TTG1, TT10, and TT1 were cloned and shown to have functions in flavonoid biosynthesis [12–19]. Yu (2013) reviewed the molecular mechanism of manupulating seed coat color in Brassica species, including the homologous TTs cloned in Brassicas [20]. Besides, molecular markers have also been developed for yellow-seeded B. rapa [21-23], B. juncea [24, 25] and B. napus. Liu et al. (2012) reported a lignin biosynthesis gene, BnCCR1, associated with yellow seed character, indicating a strong correlation between seed color and lignin content [26]. Stein et al. (2013) found that BnAHA10 had effects on seed color and lignin content [27]. Wang et al. (2017) identified 22 single nucleotide polymorphisms (SNPs) on 7 chromosomes associated with seed coat color using a genome wide association study (GWAS) [28]. Functional analysis of BnTT10 and BnTT1 revealed that they are involved in PA metabolism, lignin synthesis, seed coat pigmentation, and fatty acid (FA) biosynthesis [18, 19]. However, due to the genome complexity of *B. napus* and the sensitivity of yellow seed color to environmental influences (e.g., light, temperature, fertilizers, and harvesting time), the molecular mechanisms of this phenotype remained unclear until now [29, 30].

The short domestication history of B. napus after interspecific hybridization between B. rapa and B. oleracea and the artificial selection during rapeseed breeding have greatly narrowed the genetic background [31]. No yellow-seed germplasm has been found in natural B. napus, and most of the yellow-seeded B. napus were created by crossing between Brassica species, such as hybrids between B. napus and B. juncea, B. napus and B. carinata, B. juncea and B. oleracea [32, 33]. Wild species in Brassicaceae, such as Sinapis alba, S. arvensis, Camelina sativa, Crambe abyssinica, and Descurainia sophia, exhibit many desirable characteristics, such as yellow seed coat, high erucic acid, pod shattering resistance, high unsaturated FA contents, and resistance to various diseases and abiotic stresses [34-36]. Introducing gene resources from these wild species would help to enrich the genetic background of B. napus, accompanying desirable traits. Zhang et al. (2009) obtained yellow-seeded rapeseed from intergeneric hybrids between B. napus and D. sophia [37]. Previously, Wang et al. (2005) created somatic hybrids between *B. napus* and *S. alba* [38] and selected yellow-seeded B. napus from hybrid progenies with improved oil content and decreased antinutrients [8, 39, 40]. In the present study, we used RNA-Seq to identify the expression differences at different seed developmental stages of yellowand black-seeded B. napus, revealing the expression patterns of genes involved in various biological pathways related to seed coat pigmentation, FA biosynthesis and metabolism.

#### Results

## Transcriptome sequencing and read mapping of yellowand black-seeded *B. napus*

RNA-Seq was performed at three weeks after flowering (3 WAF), 4 WAF, 5 WAF, 6 WAF, and mature stage of yellow- and black-seeded *B. napus* to investigate the transcriptome difference that might be related to the quality variation between two rapeseeds. After quality control, ~ 30.3 to ~ 37.6 million reads from the libraries were uniquely mapped to the *B. napus* genome (Table 1). A total of 85,407 genes, including 4317 novel genes, were expressed in the developing seeds of two rapeseeds (Fig. 1, Additional file 1: Table S1). We found 52,452 and 51,777 common genes were expressed in the developing seeds of yellow and black rapeseed, respectively. In addition, 67,629 (3 WAF), 64,070 (4 WAF), 64,775 (5 WAF), 62,143(6 WAF), and 54,257 (mature stage) overlapping genes were expressed at each stage in two rapeseed lines.

 Table 1
 Summary of read mapping for RNA-seq

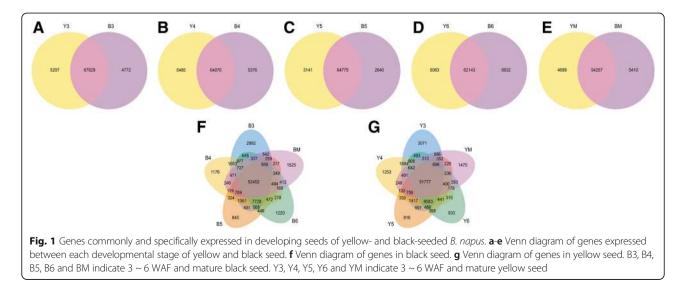
Sample name	B3	B4	B5	B6	BM	Y3	Y4	Y5	Y6	YM
Raw reads	47,536,866	42,753,820	44,341,556	51,507,518	51,272,946	48,704,282	45,694,038	44,797,562	45,996,584	52,804,244
Clean reads	45,555,018	41,801,216	40,620,518	43,833,034	48,665,348	46,813,976	43,798,322	42,843,336	43,736,106	50,268,482
Total	36,499,205	34,214,293	32,844,328	35,032,820	39,325,179	38,116,991	35,893,586	35,376,347	36,029,106	40,839,634
mapped	(80.12%)	(81.85%)	(80.86%)	(79.92%)	(80.81%)	(81.42%)	(81.95%)	(82.57%)	(82.38%)	(81.24%)
Multiple	2,175,877	2,332,136	2,561,972	3,271,514	3,151,365	2,327,452	2,287,911	2,274,889	2,451,932	3,198,280
mapped	(4.78%)	(5.58%)	(6.31%)	(7.46%)	(6.48%)	(4.97%)	(5.22%)	(5.31%)	(5.61%)	(6.36%)
Uniquely	34,323,328	31,882,157	30,282,356	31,761,306	36,173,814	35,789,539	33,605,675	33,101,458	33,577,174	37,641,354
mapped	(75.34%)	(76.27%)	(74.55%)	(72.46%)	(74.33%)	(76.45%)	(76.73%)	(77.26%)	(76.77%)	(74.88%)
Reads map	17,137,064	15,873,247	15,138,033	15,900,632	18,123,297	17,872,927	16,769,787	16,508,902	16,764,771	18,866,098
to '+'	(37.62%)	(37.97%)	(37.27%)	(36.28%)	(37.24%)	(38.18%)	(38.29%)	(38.53%)	(38.33%)	(37.53%)
Reads map	17,186,264	16,008,910	15,144,323	15,860,674	18,050,517	17,916,612	16,835,888	16,592,556	16,812,403	18,775,256
to '-'	(37.73%)	(38.3%)	(37.28%)	(36.18%)	(37.09%)	(38.27%)	(38.44%)	(38.73%)	(38.44%)	(37.35%)
Non-splice	21,266,684	21,368,313	21,381,177	22,323,651	24,534,251	22,491,873	22,291,894	24,114,006	24,559,779	26,061,989
reads	(46.68%)	(51.12%)	(52.64%)	(50.93%)	(50.41%)	(48.05%)	(50.9%)	(56.28%)	(56.15%)	(51.85%)
Splice	13,056,644	10,513,844	8,901,179	9,437,655	11,639,563	13,297,666	11,313,781	8,987,452	9,017,395	11,579,365
reads	(28.66%)	(25.15%)	(21.91%)	(21.53%)	(23.92%)	(28.41%)	(25.83%)	(20.98%)	(20.62%)	(23.04%)

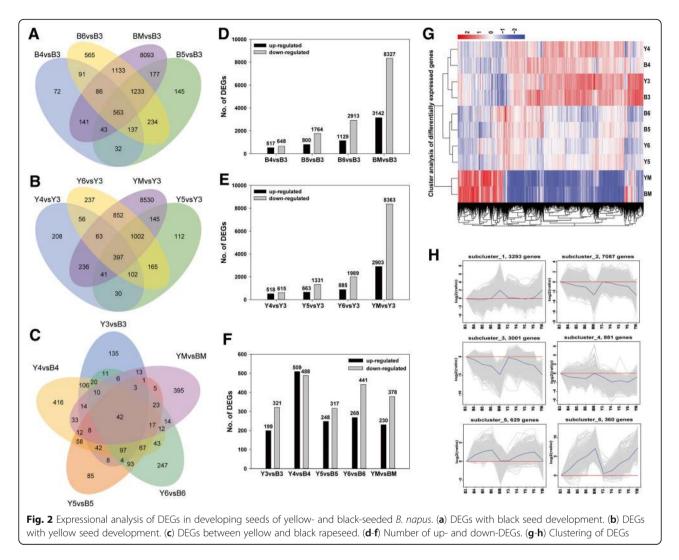
B3, B4, B5, B6 and BM indicate 3 ~ 6 WAF and mature black seed. Y3, Y4, Y5, Y6 and YM indicate 3 ~ 6 WAF and mature yellow seed

Besides, 47,912 genes were coexpressed in all the developmental stages of yellow and black rapeseed lines.

#### Identification of differentially expressed genes

Global changes in differentially expressed genes were identified using DESeq, and a total of 15,251 DEGs (including 523 novel genes) were identified among all the libraries. We found 563 common DEGs in B4 (4 WAF of black seed), B5 (5 WAF of black seed), B6 (6 WAF of black seed), and BM (mature stage of black seed) compared with B3 (3 WAF of black seed), including 141 upregulated and 331 downregulated genes at each developmental stage of the black seed. A total of 397 common DEGs were identified in Y4 (4 WAF of yellow seed), Y5 (5 WAF of yellow seed), Y6 (6 WAF of yellow seed), and YM (mature stage of yellow seed) compared with Y3 (3 WAF of yellow seed), including 92 upregulated and 220 downregulated genes throughout yellow seed development (Fig. 2a, b; Additional file 2: Table S2). Generally, 80 upregulated and 151 downregulated genes were identified during the seed development of two *B. napus* lines, which might be related to seed development and FA accumulation (Additional file 3: Table S3). Those DEGs related to cruciferin, oleosin, caleosin, late embryogenesis abundant (LEA) hydroxyproline, lipid transfer protein, myrosinase-binding protein, embryo-specific protein, and alpha-tonoplast intrinsic protein were upregulated, and those related to galactosyltransferase, glycosyl hydrolase, lipid transfer protein, basic leucine zipper protein, cytochrome P450 (CYP), laccase, chalcone synthase, delta vacuolar processing enzyme, jasmonic acid carboxyl





methyltransferase, and senescence were downregulated with seed development. In addition, 42 DEGs were identified between yellow and black rapeseeds at each stage, including 11 upregulated and 31 downregulated genes in yellow-seeded B. napus (Fig. 2c, Additional file 4: Table S4). These common DEGs were mainly related to the RNA-binding family protein, nascent polypeptide-associated complex (NAC), calcium binding EF-hand family protein, and metallothionein protein that were upregulated and dormancy/auxin associated protein, S-adenosylmethionine synthetase, pectin lyase-like superfamily protein, insulinase family protein that were downregulated in all stages of yellow seed compared with black seed. The number of DEGs increased with seed development in both rapeseed lines, whereas the downregulated DEGs at the mature stage dramatically increased to  $\sim$ 8000 (Fig. 2d, e). The up-regulated and downregulated DEGs between Y4 and B4 were higher than those in the other stages of the two rapeseed lines (Fig. 2f). Hierarchical cluster analysis of all the DEGs was performed using the log<sub>10</sub>(RPKM+1) value (Fig. 2g). H-cluster and SOM-cluster

of the DEGs from five developmental stages of two rapeseed lines were also performed using the log<sub>2</sub>(Fold change), and the DEGs in all the clusters showed similar patterns between two rapeseed lines, except for subcluster\_1\_1, which includes DEGs with significant differences between Y4 and B4 (Fig. 2h, Additional file 5: Figure S1).

#### **Enrichment analysis of DEGs**

To acknowledge the putative functions and pathways of the DEGs, GO and KEGG enrichment analysis of DEGs was proceeded with GOseq and KOBAS 2.0. Among all the identified DEGs, 11,772 were annotated with GO terms and assigned to three categories: cellular component, biological process, and molecular function. Most of the upand downregulated DEGs between the same stage of yellow and black seeds were assigned to catalytic activity, binding, metabolic process, cellular process, followed by cell, cell part, membrane, organelle part, macromolecular complex, regulation of biological process, and biological regulation (Fig. 3, Additional file 6: Table S5). In detail, among all the

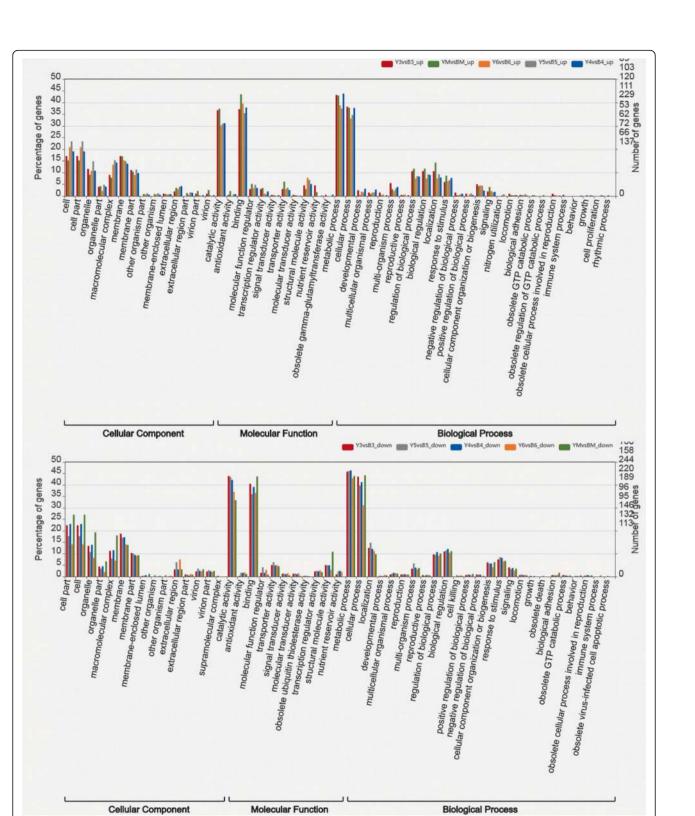


Fig. 3 GO enrichment of up- and down-DEGs between yellow- and black-seeded B. napus

downregulated genes (1435 out of 1945 with GO annotations) between yellow and black rapeseed, 425, 39, 211, 89, and 65 DEGs were assigned to biosynthetic process (GO: 0009058), pigment metabolic process (GO: 0042440), oxidation-reduction process (GO: 0055114), cellular response to stimulus (GO: 0051716), and signal transduction

(GO: 0007165), respectively. In contrast, 181 upregulated genes between yellow and black seed were assigned to biosynthetic process (GO: 0009058), 95 up-DEGs related to oxidation-reduction process (GO: 0055114), and 181 up-DEGs related to biosynthetic process (GO: 0009058).

KEGG pathway analysis showed that flavonoid biosynthesis was most significantly enriched with downregulated DEGs in comparisons of Y3 vs B3, Y4 vs B4, Y5 vs B5, and Y6 vs B6. Phenylpropanoid biosynthesis was also enriched with downregulated genes between the same stages of the two rapeseed lines, except for the mature stage. Phenylalanine metabolism, flavone and flavonol biosynthesis was enriched with downregulated DEGs in comparisons of Y3 vs B3 and Y4 vs B4 (Additional file 7: Figure S2). In contrast, the upregulated DEGs between yellow and black rapeseed were most significantly enriched in photosynthesis, followed by carbon metabolism, FA biosynthesis and metabolism (Additional file 8: Figure S3, Additional file 9: Table S6). The pathway enrichment analysis helps to determine the functions of DEGs and complemented secondary metabolisms that were not specified by GO terms. Using the log<sub>2</sub>(Fold change) values between yellow and black rapeseed (Y3 vs. B3, Y4 vs. B4, Y5 vs. B5, Y6 vs. B6, and YM vs. BM), we assigned all the DEGs to all the metabolism pathways using the Arabidopsis TAIR9 version as mapping reference. We also found many DEGs were enriched in metabolism (flavonoids, terpenes, phenylpropanoids and phenolics) and lipids (Additional file 10: Figure S4).

#### DEGs associated with flavonoid and lignin biosynthesis

As mentioned above, yellow seed color is associated with flavonoid and lignin biosynthesis. We found 77 DEGs related to phenylpropanoid and flavonoid biosynthesis and the phenylalanine metabolic pathway. As shown in Fig. 4, genes encoding 4-coumarate CoA ligase (4CL), cinnamate 4-hydroxylase (C4H), and phenylalanine ammonia-lyase (PAL) were downregulated in yellow rapeseed compared with black seed, except for a homolog of PAL (BnaC05g42780D). These genes play important roles in lignin biosynthesis, and the expression changes should be related to the reduced lignin in yellow seed. Genes involved in the flavonoid pathway were also downregulated in yellow seed compared to black seed, including BAN, CHI/TT5, DFR, F3H, FLS, LDOX, PAP, CHS/TT4, TT5, bHLH/TT8, WD40, and MYB, which participate in the biosynthesis of chalcones, flavanones, flavonols, anthocya-PAs. However, we found a MATE nins, and (BnaC01g40630D) and *UGT84A1* (BnaA01g18540D) were upregulated at the early developmental stages of yellow seed compared with black seed. Comparing with other two down-regulated MATEs in yellow-seeded B. napus, BnaA01g24940D (a homologous of AT3G03620) and BnaA07g18120D (a homologous of AtTT12), we found BnaC01g40630D with lower expression throughout rapeseed development than that of BnaA01g24940D and BnaA07g18120D. Expressional differences of TCPs and CYPs were also identified, which might be related to flavonoid biosynthesis, since TCP could interact with R2R3-MYB [41], and CYPs (e.g., CYP71/75/93/81) have been reported to function in the biosynthesis of anthocyanin pigments and condensed tannins [42]. In addition, we compared the expression pattern of all the genes involved in secondary metabolism and found DEGs encoding peroxidase superfamily protein (BnaA02g02010D, BnaC06g32660D, BnaA02g14050D, and BnaC06g21080D), glycosyl hydrolase family protein (BnaUnng02770D), beta glucosidase 19/29/ 25 (BnaA06g09700D, BnaA05g04040D, BnaC05g11190D, BnaC01g43700D, and BnaC01g40610D), thioglucoside glucohydrolase 1 (BnaCnng53320D) were also assigned to phenylpropanoid biosynthesis and the phenylalanine metabolic pathway (Additional file 11: Figure S5). Generally, most of the genes involved in secondary metabolism were downregulated in yellow seed compared with black seed.

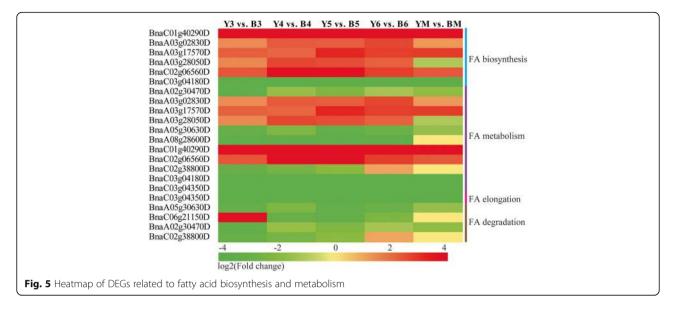
# DEGs associated with fatty acid biosynthesis and metabolism

We found that genes involved in FA biosynthesis, metabolism, and degradation were also changed (Fig. 5). For instance, five up-DEGs in yellow seed were involved in FA biosynthesis, encoding an NAD(P)-binding Rossmann-fold superfamily protein (BnaC01g40290D), a thioesterase superfamily protein (BnaA03g02830D), acetyl Coenzyme A carboxylase carboxyltransferase (BnaA03g17570D), stearoyl-acyl-carrier-protein desaturase (BnaA03g28050D), and chloroplastic acetylcoenzyme A carboxylase 1 (BnaC02g06560D). However, another thioesterase superfamily homolog of the protein (BnaC03g04180D) was downregulated in yellow seed compared with black seed. Five up-DEGs (BnaA03g02830D, BnaA03g17570D, BnaA03g28050D, BnaC01g40290D, and BnaC02g06560D) and six down-DEGs (BnaA02g30470D, BnaA05g30630D, BnaA08g28600D, BnaC02g38800D, Bna C03g04180D, and BnaC03g04350D) were involved in FA metabolism. BnaC03g04350D was also related to FA elongation. In addition, four down-DEGs (BnaC06g21150D, BnaA02g30470D, BnaA05g30630D, and BnaC02g38800D) were related to FA degradation. These DEGs might be related to the different oil content between yellow- and black-seeded B. napus.

## Real-time qPCR validates gene expression profiles

To verify the sequencing results, DEGs involved in flavonoid and lignin biosynthesis, FA biosynthesis and metabolism were randomly selected for qPCR detection, including BnaC01g40290D encoding NAD(P)-binding Rossmann-fold superfamily protein/short-chain dehydrogenase/reductase (SDR), BnaA03g17570D encoding acetyl CoA carboxylase carboxyltransferase alpha subunit (CAC3), BnaC03g04350D

XV x. B3       Y4 xy. B4       Y3 xy. B5       Y6 xy. B6       Y4 xy. B4         BAN       BraA01660000       BAN       BraA01660000       BAN       BraA01660000         BAN       BraA01660000       BAN       BraA01660000       BAN       BraA01660000         BAN       BraA01670000       BAN       BraA01670000       BAN       BraA01670000         BAN       BraA01670000       BAN       BraA01670000       BAN       BraA01670000         BHILH       BraA01670000       BRA00670000       BRA00670000       BRA00670000       BRA00670000         CHI       BraC02670000       BRA00670000       BRA00670000       BRA00670000       BRA00670000         PALI       BraC02670000       BRA00670000       BRA00670000       BRA00670000       BRA00670000         PALI       BraC0267000       BRA00670000       BRA00670000       BRA00670000       BRA00670000         PALI       BraC0267000       BRA00670000       BRA00670000       BRA00670000       BRA00670000         PALI       BraC0267000       BRA00670000       BRA00670000       BRA00670000       BRA006700000         PARS       BraA01670000       BRA006700000       BRA006700000       BRA006700000       BRA0067000000       BRA0067000000									
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BAN       Bmc(CH29900)         HILH       BmaA01242000         VIP       BmaA01242000         CH       BmaC02419100         FH       BmaC02419100									
HHLH       Bra.A012/47020         KZP       Bra.A012/47020         KZP       Bra.A023/47020         C4H       Bra.A023/47020         C4H       Bra.A023/47020         C4H       Bra.C0250000         C4H       Bra.C0250000         C4H       Bra.C0250000         C4H       Bra.C0257000         DFR       Bra.C0257000         FSH       Bra.C0257000         LDXX       Bra.C0257000         PAL1       Bra.C0257000         PAL2<									
bill.H       Bia.012/23/200         C4H       Bia.02/23/200         F3H       Bia.02/23/200         C4H       Bia.02/23/200         PAL       Bia.02/23/200         P				1				and the second se	
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CHI BracObj 50500 DFR BracObj 17100 F3H BracObj 17100 F3H BracObj 1700 F1SH BracObj		В	naA05g11950D						
CHI Bra.4069(17150) DFR Bra.4069(17150) F3H Bra.4069(22400) F1S1 Bra.4069(22400) F1S1 Bra.4069(2300) LDOX Bra.C019(4500) LDOX Bra.C019(4500) DFR Bra.2059(7700) NYB107 Bra.4069(23500) PAL1 Bra.4069(23500) PAL1 Bra.4069(23500) PAL1 Bra.4069(23500) PAL2 Bra.405(24600) PAL2 Bra.405(24600) PAL4 Bra.4069(23500) T14 Bra.4069(23500) T15 Bra.406(24500) T14 Bra.4069(23500) T15 Bra.406(24500) T15 Bra.406(24500) T16 Bra.406(24500) T17 Bra.406(24500) T17 Bra.406(24500) T17 Bra.406(24500) T18 Bra.406(24500) T19 Bra.406(24500) T19 Bra.406(24500) T19 Bra.406(24500) T19 Bra.406(24500) T19 Bra.406(24500) T19 Bra.406(24500) T19 Bra.406(24500) T19 Bra.406(24500) CYP7A7 Bra.406(24500) CYP7A52 Bra.406(24500) CYP7A542 Bra.405(24500) CYP7A542 Bra.405(24500) CYP7A54 Bra.405(2									
DFR       Bma.O02917500         F3H       Bma.O02917600         F1SI       Bma.O02917600         LBD4H       Bma.O0297600         LD0X       Bma.O02977600         LD0X       Bma.O02977700         PAL1       Bma.O02977700         PAL2       Bma.O02977700         PAL3       Bma.O02977700         PAL4       Bma.O02977700         PAL4       Bma.O02977700         PAL52       Bma.O0297700         PAL4       Bma.O02978700         PAL4       Bma.O02978700         TT4       Bma.O02978700         TT4       Bma.O02978700         TT4       Bma.O02978700         TT4       Bma.O02978700         TT4       Bma.O02978700									
DFR       Brax 009217500         F3H       Brax 00922800         FLS1       Brax 01922800         FLS1       Brax 01923900         LDOX       Brax 01923900         LDOX       Brax 01923900         NYB 101       Brax 01923900         NYB 101       Brax 01923900         NYB 101       Brax 01923900         NYB 111       Brax 01923900         PAL1       Brax 01924900         PAL3       Brax 01924500         PAP85       Brax 01924500         PAP85       Brax 01924500         PAP85       Brax 01924500         TT4       Brax 029245700         TT5       Brax 029245700         UGT71C2       Brax 04921500         UGT71C2       Brax 04921500         UGT71C4       Brax 02925100         UGT71C4       Brax 02925100         UGT71C4       Brax 02925100         UGT71C4									
F3H       Bma.Obg2300D         FLS1       Bma.Obg2300D         LBD41       Bma.Obg3301D         LD0X       Bma.Obg3301D         DL0X       Bma.Obg3301D         MYB107       Bma.Obg3500D         PAL1       Bma.Obg3500D         PAL3       Bma.Obg3520D         TH4       Bma.Obg3520D         TH4       Bma.Obg3530D         TT4       Bma.Obg3530D         CP7-1       Bma.Obg3530D         CP7-1									
F3H       Brac Obje 224000         FLS1       Brac Obje 230100         LBD41       Brac Obje 230100         LD0X       Brac Obje 230100         MYB101       Brac Obje 230100         YPB118       Brac Obje 230100         PAL1       Brac Advig 12400         PAL2       Brac Advig 12400         PAL3       Brac Obje 1200         TT4       Brac Obje 1200         UGT717C       Brac Obje 1200         UGT717C4       Brac Obje 1200								_	
FLS1       BmaC0923000         FLS1       BmaC02933000         LDDX       BmaC02933000         LDDX       BmaC02933000         LDDX       BmaC02933000         MYB107       BmaC02933000         PAL1       BmaC02937000         PAL3       BmaC02937000         PAP85       BmaC02937000         PAP85       BmaC02937000         T4       BmaC02937000         T74       BmaC02937000         T74       BmaC									
FLS1       Bac(02)817300         LBD41       Bac(02)817300         LD0X       Bac(02)817500         LD0X       Bac(02)817500         LD0X       Bac(02)817500         MYB107       Bac(02)817500         PAL1       Bac(02)817500         PAL1       Bac(02)817500         PAL1       Bac(02)817500         PAL1       Bac(02)817500         PAL1       Bac(02)817600         PAL1       Bac(02)817600         PAL3       Bac(02)817600         PAL4       Bac(02)817600         PA14       Bac(02)817600         PA14       Bac(02)81760         TT4       Bac(02)81760         TCP-1       Bac(02)81760         TCP-1       Bac(02)81760         CYP7121       Bac(02)81760         CYP7121       Bac(02)81760         CYP7121       Bac(02)81760         CYP7121       Bac(02)81760				_					
LEDAH Buc(20)2330100 LDOX Buc(20)237000 WHB107 Buc(20)237000 PAL1 Buc(20)237000 PAL1 Buc(20)237000 PAL1 Buc(20)21200 PAL2 Buc(20)21200 PAL3 Buc(20)21200 PAL3 Buc(20)21200 PAL4 Buc(20)2100 PAL4 Buc(20)2100 PAL4 Buc(20)2100 PAL4 Buc(20)21000 PAL4 Buc(20)2100 PAL4 Buc(20)2100 PAL4 Buc(20)2100 PAL4 Buc(									
LDOX BmaX0g12300 LDOX BmaX0g237000 WHB107 BmaXmg12300 PAL1 BmaX0g237000 PAL1 BmaX0g212300 PAL1 BmaX0g212300 PAL1 BmaX0g212300 PAL3 BmaX0g237000 PAL4 BmaX0g237000 PAL4 BmaX0g237000 PAL4 BmaX0g237000 TT4 BmaX0g23700 TT4 BmaX0g23700 TT4 BmaX0g23700 TT4 BmaX0g23700 TT4 BmaX0g23700 TT9 BmaX0g23700 TT9 BmaX0g23700 TCP-1 BmaX0g23700 TCP-1 BmaX0g23700 TCP-1 BmaX0g23700 TCP-1 BmaX0g23700 CYP705A22 BmaX6g19000 CYP71313 BmaX0g23700 CYP71313 BmaX0g23700 CYP73143 BmaX0g23									
LDOX BrauX05g45100 DDX BrauX05g45100 PALI BrauX04212400 PALI BrauX04212400 PALI BrauX04212400 PALI BrauX04212400 PAL2 BrauX01245600 PAP35 BrauX012245600 PAP85 BrauX0122									
LDOX BuaCong 32000 MYBIIS BuaCog 37000 PALI BuaCog 37000 PALI BuaCog 37000 PALI BuaCog 37000 PALI BuaCog 37000 PALI BuaCog 37000 PALI BuaCog 321200 PALI BuaCog 324700 PALI BuaCog 324700 PALA BuaCog 324700 PALA BuaCog 32500 TT4 BuaCog 32500 TT4 BuaCog 32500 TT4 BuaCog 32500 TT4 BuaCog 32500 TT4 BuaCog 32500 TT4 BuaCog 32500 TT8 BuaCog 32700 TT8 BuaCog 32700 TT8 BuaCog 32700 TT8 BuaCog 32700 CG 773C2 BuaCog 32600 CG 773C2 BuaCog 32600 CG 773C2 BuaCog 32600 CG 773C2 BuaCog 32500 TCP-1 BuaCog 32500 CG 773C2 BuaCog 32500 CG 7775C3 BuaCog 32500 CG 7775C									
MYB101       BuaAcugi 52000         PAL1       BuaC029/07000         PAL1       BuaC029/07000         PAL1       BuaC029/07000         PAL1       BuaC029/07000         PAL2       BuaA012/06000         PAL4       BuaC029/07000         PAL5       BuaA012/06000         PAL4       BuaC029/07000         PAF85       BuaA012/05000         TF4       BuaC029/07000         TF4       BuaC029/07000         TF4       BuaC029/07000         TF4       BuaC029/07000         TF4       BuaC029/07000         TF5       BuaC09/02/0700         TF4       BuaC029/0700         TF4       BuaC029/0700         TF5       BuaC09/02/0700         TF8       BuaC09/02/0700         TF8       BuaC09/02/0700         UGTFX4A       BuaC019/02/0700         UGTFX4A       BuaC019/07000         UCF71A <td></td> <td></td> <td></td> <td></td> <td></td> <td></td> <td></td> <td>Concession of the local division of the loca</td> <td></td>								Concession of the local division of the loca	
MYB118       BmaC02g37090D         PAL1       BmaC02g3707D         PAL1       BmaC02g3707D         PAL1       BmaC02g3707D         PAL1       BmaC02g3707D         PAL1       BmaC02g3707D         PAL1       BmaC02g3707D         PAL2       BmaC01g4560D         PAL4       BmaC02g3700D         PAL5       BmaC01g2450D         PAP85       BmaC01g2450D         TF4       BmaC02g3700D         TF4       BmaC02g470D         TF4       BmaC02g4200D         TF3       BmaC02g2470D         TF3       BmaC02g2470D         TF3       BmaC02g2470D         TF3       BmaC02g2470D         TF3       BmaC02g2470D         WD40       BmaC02g2470D         UGT74C4       BmaA01g2490D         WGT71C4       BmaA02g2310D         UGT74C4       BmaA02g230D         TCP-1       BmaC01g4350D         TCP-1       BmaC01g4350D         TCP-1       BmaC01g4350D         CYP176A2       BmaC01g4350D         CYP176A22       BmaC01g4350D         CYP176A22       BmaC01g4350D         CYP176A22       BmaC01g4350D								-	
PAL1 BmacOug/9100 PAL1 BmacOug/9100 PAL1 BmacOug/92400 PAL2 BmacOug/92400 PAL4 BmacOug/92400 PAL4 BmacOug/92400 PAP85 BmacOug/92600 TT4 BmacOug/92000 TT4 BmacOug/92000 TT4 BmacOug/92000 TT5 BmacOug/92100 WD40 BmacOug/9100 WD40 B				No. of Concession, name					
PALI       BmaAO4g212400         PALI       BmaAO4g212400         PALI       BmaAO4g212400         PALA       BmaAO5g284700         PALA       BmaAO1g246600         PAPRS       BmaAO1g246600         PAPRS       BmaAO1g246600         PAPRS       BmaAO1g246600         TT4       BmaAO1g24600         TT4       BmaAO1g24600         TT4       BmaAO1g24600         TT4       BmaAO1g24600         TT4       BmaAO1g24600         TT5       BmaCO2g28700         TT5       BmaCO2g280000         WD40       BmaAO1g228100         WD40       BmaAO1g228100         WD40       BmaAO1g228100         UGTT3C2       BmaAO1g228100         UGTT4LA       BmaAO1g228100         UGTT4LA       BmaAO1g228100         UGTT4LA       BmaAO1g228100         UGTT4LA       BmaAO1g228100         UGTT4LA       BmaAO1g228100         UGTT4LA       BmaAO1g23800         TCP-1       BmaAO1g238100         TCP-1       BmaAO1g23800         CYP102L       BmaAO2g280100         CYP113       BmaAO2g380100         CYP1242       BmaA								and the second second	
PALI       BmaA04212400         PALZ       BmaA072160600         PALA       BmaA072160600         PALA       BmaA072160600         PALA       BmaA025427800         PAP85       BmaA01245600         TT4       BmaC059427800         TT4       BmaC02943200         TT4       BmaC02943200         TT4       BmaC02943200         TT4       BmaC02923700         TT4       BmaC02924700         TT4       BmaC02924700         TT8       BmaC029248700         WD40       BmaC02922100         WD40       BmaC02922100         WD40       BmaC02922100         WD40       BmaC02923100         WD40       BmaC029248700         UGT73C2       BmaA04210900         UGT74C4       BmaC029238100         UGT74C4       BmaC02923800         TCP-1       BmaC0292383800         TCP-1       BmaC029238100         CYP105A22       BmaA05219800         CYP113       BmaA02923800         CYP124       BmaA02923800         CYP134       BmaA02923800         CYP143       BmaA02923800         CYP1705A22       BmaA05925800									
PALI BmaA07e12400 PAL3 BmaA07e12400 PAL4 BmaA05g284700 PAL4 BmaA05g284700 PAP85 BmaA01g24500 PAP85 BmaA01g24500 TT4 BmaA02g83700 TT4 BmaC03g61200 TT4 BmaC03g61200 TT5 BmaC03g264700 TT8 BmaA09g228100 UGT71C1 BmaC03g209100 WD40 BmaA02g28100 UGT71C2 BmaA08g12900 UGT71C4 BmaA08g218100 UGT71C4 BmaA08g218100 UGT71C4 BmaA08g218100 UGT71C4 BmaA08g218100 CTP-1 BmaA08g218100 CTPF1A BmaA08g								The second s	
PAL2       BmaA072160600         PAL4       BmaA052284700         PAP85       BmaA012245600         PAP85       BmaA012245600         TT4       BmaC02943200         TT4       BmaC02943200         TT4       BmaC02943200         TT4       BmaC029248700         TT4       BmaC029248700         TT8       BmaC029248700         TT8       BmaC029228100         WD40       BmaA029228100         WD40       BmaA029228100         WD40       BmaA02928100         UGT73C2       BmaA04924900         UGT73C2       BmaA04924900         UGT74C4       BmaC059219500         TCP-1       BmaC059219500         TCP-1       BmaC059219500         TCP-1       BmaC059219500         CYP105A22       BmaA05219500         CYP114       BmaC059219500         CYP134       BmaA05225500         CYP143       BmaA0525500         CYP705A22       BmaA0525500         CYP705A22       BmaA0525500         CYP705A22       BmaA0525500         CYP776A1       BmaA0525500         CYP776A1       BmaA0525500         CYP776A2 <t< td=""><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td>and the second second</td><td></td></t<>								and the second second	
PAL4       Bmax05g24700D         PAP85       Bmax01g24560D         PAP85       Bmax01g24560D         T14       BmaxC0g42520D         T14       BmaxC0g24570D         T14       BmaxC0g24570D         T14       BmaxC0g26373D         T14       BmaxC0g26373D         T14       BmaxC0g26370D         T15       BmaxC0g26370D         T18       BmaxC0g26370D         T18       BmaxC0g26370D         WD40       BmaxC0g24870D         WD40       BmaxC0g24870D         WD40       BmaxC0g24870D         UGT71C4       BmaxC0g24870D         UGT71C4       BmaxC0g24870D         UGT71C4       BmaxC0g24970D         UGT71C4       BmaxC0g2530D         TCP-1       BmaxC0g23580D         TCP-1       BmaxC0g23580D         CYP105A22       BmaxA0g23580D         CYP705A22       BmaxA0g23580D         CYP705A22       BmaxA0g23580D         CYP705A22       BmaxA0g2580D         CYP705A22       BmaxA0g2580D         CYP705A22       BmaxA0g55330D         CYP705A23       BmaxA0g55330D         CYP775A3       BmaxA0g55330D								i i contra a	
PAL4       BmaC05_92780D         PAP85       BmaA01g24560D         PAP85       BmaA01g24560D         T14       BmaC02g38730D         T14       BmaC02g05070D         T15       BmaC02g05070D         T18       BmaC09g24870D         WD40       BmaC02g05070D         T18       BmaC09g24870D         WD40       BmaC02g05070D         T18       BmaC09g24870D         WD40       BmaC0g22810D         UGT71C4       BmaC01g4030D         UGT71C4       BmaC01g4030D         UGT71C4       BmaC01g4030D         UGT71C4       BmaC01g4030D         UCT71C4       BmaC01g4030D         TCP-1       BmaC0g23530D         TCP-1       BmaC03g2350D         CYP10       BmaC03g2530D         CYP1313       BmaC03g2530D         CYP705A22       BmaA0g2530D         CYP72A1       BmaC03g2530D         CYP72A1       BmaC03g2530D         CYP72A5       BmaC03g2530D         CYP72A5       BmaC05g2530D         CYP73A5       BmaC05g2530D         CYP74A5       BmaC05g2530D         CYP74A7       BmaC05g2530D         CYP74A7									
PAP85       BmaA01 (2450D)         T14       BmaC094250D         T14       BmaC094250D         T14       BmaC020507D         T14       BmaC020507D         T15       BmaC0820507D         T15       BmaC0820507D         T18       BmaC0922487DD         WD40       BmaC020910D         WD40       BmaC0209310D         WD40       BmaC032057DD         UCGT73C2       BmaA0422190DD         UCGT74C4       BmaC032057DD         TCP-1       BmaC032057DD         TCP-1       BmaC032075DD         TCP-1       BmaC032075DD         CYP161G       BmaC012403DD         CYP176A1       BmaC032075DD         CYP176A2       BmaC032075DD         CYP176A2       BmaC032075DD         CYP176A2       BmaC032075DD         CYP176A2       BmaC0320375DD         CYP176A2       BmaC0320375DD         CYP176A2       BmaC0320375DD         CYP176A3       BmaC0320375DD         CYP176A4       BmaC0320375DD         CYP176A2       BmaC0320375DD         CYP176A3       BmaC0320375DD         CYP176A4       BmaC0320375DD         CYP176A4<									
TT4       BnaA 10g 1970D         TT4       BnaC 02g 38730D         TT4       BnaC 02g 38730D         TT4       BnaC 02g 0570D         TT5       BnaC 08g 26020D         TT8       BnaC 08g 26020D         TT8       BnaC 08g 2670D         WD40       BnaA 09g 22810D         WD40       BnaA 09g 22810D         WD40       BnaA 01g 24940D         MATE       BnaC 01g 49630D         UGT73C2       BnaA 01g 18540D         UGT71C4       BnaC 08g 4520D         UGT84A1       BnaA 01g 18540D         CP-1       BnaC 01g 40530D         TCP-1       BnaC 01g 40530D         CYP1       BnaC 01g 40530D         CYP1       BnaC 01g 4052D         CYP1 BanaC 03g 5530D         CYP1A       BnaC 00g 4072D         CYP1A1       BnaC 00g 4072D         CYP1A21       BnaC 00g 4072D         CYP1A32       BnaA 00g 25300D         CYP1A452       BnaA 00g 25330D <t< td=""><td>PAP85</td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td></t<>	PAP85								
TT4       BnaC002943200         TT4       BnaC02295700         TT4       BnaC02205700         TT5       BnaC08240200         TT8       BnaC09224100         WD40       BnaC089437400         WD40       BnaC089437400         WT5       BnaC089437400         WT6       BnaC089437400         WT73C2       BnaA012249400         UGT71C4       BnaC089452000         UGT71C5       BnaA01824900         UGT73C2       BnaA01824900         UGT73C2       BnaA01824900         UGT73C2       BnaA01824900         UGT73C4       BnaC089452000         UGT81A41       BnaA012490500         TCP-1       BnaC089452000         UGT81A41       BnaA019249000         TCP-1       BnaC05925700         TCP-1       BnaC03925700         CYP1       BnaC03925700         CYP15161       BnaC03925700         CYP161       BnaC03925700         CYP15161       BnaC03925700         CYP1522       BnaA05219900         CYP15161       BnaC03925700         CYP15161       BnaC03925700         CYP172A1       BnaC09210200         CYP75A22 <td>PAP85</td> <td>В</td> <td>naA01g24560D</td> <td></td> <td></td> <td></td> <td></td> <td></td> <td></td>	PAP85	В	naA01g24560D						
TT4       BnaC02g38730D         TT4       BnaC02g05070D         TT5       BnaC08g2000D         TT8       BnaC09g24870D         WD40       BnaAC0g20910D         WD40       BnaAC0g20910D         WD40       BnaAC0g20910D         WD40       BnaAC0g20910D         WD40       BnaAC0g20910D         WD41       BnaAC0g20910D         UGT71C2       BnaAC0g24870D         UGT71C4       BnaC08g4520D         UGT84A1       BnaAO1g23810D         TCP-1       BnaAO3g2280D         TCP-1       BnaAO3g2280D         TCP-1       BnaAO3g29270D         TCP-1       BnaAO3g29270D         TCP-1       BnaAO3g29270D         TCP-1       BnaAO3g29270D         CYP1       BnaAO3g29270D         CYP1       BnaAO3g29270D         CYP1       BnaAO3g29270D         CYP1       BnaAO3g29270D         CYP1       BnaAO3g29270D         CYP1       BnaAO3g29270D         CYP1A1       BnaAO9g39310D         CYP705A22       BnaAO5g19010D         CYP705A22       BnaAO5g19010D         CYP705A22       BnaAO5g2930D         CYP771A1	TT4	B	naA10g19670D		-				
TT4       BmaCO3206120D         TT5       BmaCO32029070D         TT5       BmaCO32029070D         TT8       BmaAO3229070D         WD40       BmaCO32042940D         WD40       BmaCO3204940D         WD41       BmaAO1224940D         WD42       BmaAO1224940D         WD43       BmaAO1224940D         WT73C2       BmaAO1224940D         UGT71AC1       BmaAO128520D         UGT71AC2       BmaAO12830D         TCP-1       BmaAO12803DD         TCP-1       BmaAO12803DD         TCP-1       BmaAO12803DD         TCP-1       BmaAO32293DD         CYP1       BmaAO32293DD         CYP1       BmaAO32293DD         CYP1       BmaAO32293DD         CYP1A       BmaAO32293DD         CYP705A22       BmaAO52190DD         CYP7	TT4	B	naC09g43250D						
TT4       BmaC0826020D         TT8       BmaC0822620D         TT8       BmaC0822810D         WD40       BmaA022201D         WD40       BmaA022091D0         WD40       BmaA022091D0         WD40       BmaA022091D0         WD40       BmaA022091D0         WD41       BmaA0124940D         WATE       BmaA0124940D         UGT73C2       BmaA042190D         UGT74C4       BmaA02842190D         UGT74C4       BmaA0282219D         TCP-1       BmaC01264330D         TCP-1       BmaC012632350D         TCP-1       BmaC012632350D         TCP-1       BmaC0360160D         CYP1       BmaA03229270D         TCP-1       BmaC0360160D         CYP1042       BmaA03229270D         CYP705A22       BmaA0521950D         CYP78A9       BmaC0926510D         CYP78A9       BmaA02825350D         CYP78A9 <td></td> <td>B</td> <td>naC02g38730D</td> <td></td> <td></td> <td></td> <td></td> <td></td> <td></td>		B	naC02g38730D						
TT5       BraC0822800D         TT8       BraA0922810D         WD40       BraC0849370D         WD40       BraC0849370D         WD40       BraC0849370D         WD40       BraC0124003D         WT7752       BraA0122900D         UGT73C2       BraA0121990D         UGT73C2       BraA07233810D         TCP-1       BraC0124050D         UGT84A1       BraA0121990D         TCP-1       BraC0124050D         CYP1       BraC0320350D         TCP-1       BraC0124050D         CYP1       BraC0320350D         CYP1       BraC0320350D         CYP151G1       BraA0322580D         CYP705A22       BraA0521930D         CYP72A7       BraC012470D         CYP78A9       BraC0831760D									
TT8       BmaC0922810D         WD40       BmaA02200910D         WD40       BmaC0843740D         MATE       BmaC012040030D         UGT73C2       BmaA01224940D         MATE       BmaC01204030D         UGT73C2       BmaA01224940D         UGT73C2       BmaA01224940D         UGT73C2       BmaA01224940D         UGT73C2       BmaA0122490D         UGT84A1       BmaA0123290D         TCP-1       BmaC032270D         TCP-1       BmaC032270D         TCP-1       BmaC0320530D         CYP1       BmaC03260160D         CYP1       BmaC03260750D         CYP1       BmaC03260750D         CYP1       BmaC03260750D         CYP1       BmaC03260750D         CYP702A1       BmaC03260750D         CYP702A1       BmaC03260750D         CYP702A1       BmaC03260750D         CYP705A22       BmaA06225320D         CYP705A22       BmaA06225320D         CYP705A22       BmaA06225320D         CYP705A22       BmaA06225320D         CYP76C4       BmaC0925370D         CYP77A7       BmaC0126370D         CYP778A9       BmaC0925370D									
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tmap of DEGs related to flavonoid and lignin biosynthesis	Heatmap of DEGs related to a	lavono	id and lignin bi	osynthesis					

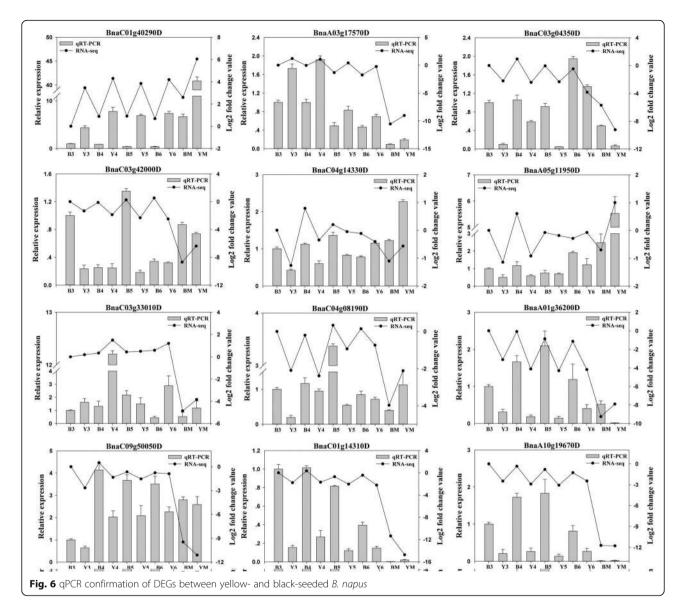


encoding 3-hydroxyacyl-CoA dehydratase PASTICCINO 2 (PAS2), 4CL (BnaC03g42000D), C4H (BnaC04g14330D, BnaA05g11950D), LBD41 (BnaC03g33010D), PAL (BnaC04g08190D), BAN (BnaA01g36200D), CHI (BnaC09g50050D), LDOX (BnaC01g14310D) and CHS (BnaA10g19670D). The results showed that the qPCR data of the DEGs were in accordance with the sequencing results throughout seed development of yellow- and black-seeded B. napus (Fig. 6).

#### Discussion

Due to the important economic value of rapeseed worldwide, research on improving seed quality has been carried out over the past half century, including reducing the erucic acid and glucosinolate contents and increasing the oleic acid content [43, 44]. However, breeding of rapeseed has been greatly hindered by the narrow genetic background either due to the short history or the artificial selection [31]. Previously, Wang et al. created yellow-seeded B. napus from the backcrossing progenies of B. napus-S. alba hybrids, including S. alba specific fragments in the 38 chromosomes [8, 34, 45]. RNA-Seq is a reliable method in analyzing the gene expressional level on the whole transcriptome level, and it also helps to reveal the expressional differences among different samples, which could somehow explain the different characteristics among samples. In the past decade, RNA-Seq has been broadly applied in expressional comparison among plant samples, and in finding alternative splicing, novel transcripts, SNP and InDels [46-48]. To identify the gene expression differences related to yellow seed character and accompanying quality variations, we carried out comparative transcriptome analysis between different developmental stages of yellowand black-seeded B. napus. As reported by Qu et al. (2013), accumulation of polymeric phenolic compounds were similar in yellow and black rapeseeds, but the flavonoids were more accumulated in black seed since 3 WAF [49]. Thus, we collected the seeds at 3 WAF, 4 WAF, 5 WAF, 6 WAF and mature stage for RNA-Seq analysis. We found a total of 39,632 SNPs in ten libraries, including 1142 SNPs common in five developmental stages of black seed and 1543 SNPs common in five developmental stages of yellow seed, indicating that genomic differences exist between yellow- and black-seeded B. napus. We found a total of 15,251 DEGs among all the libraries, including 80 upregulated and 151 downregulated genes identified throughout yellow and black seed development, which might be related to seed development and FA accumulation (Additional file 2: Table S2). Regarding the DEGs between yellow and black rapeseed, we found that most were annotated to biosynthetic, pigment metabolic, and oxidation-reduction processes, such as flavonoid and phenylpropanoid biosynthesis, phenylalanine metabolism, flavone and flavonol biosynthesis, and FA biosynthesis and metabolism (Additional file 8: Figure S3, Additional file 9: Table S6). This agreed with Hong et al. who found that the downregulated DEGs in yellow seed coats were enriched in phenylpropanoid and flavonoid biosynthesis using yellow- and brown-seeded near-isogenic lines (NILs) of rapeseed as research materials [50]. Similar expression changes in TT genes have been reported in *B. juncea* seed coat [51].

Flavonoid biosynthesis has been considered to be the main pathway related to plant pigmentation, and *TT* genes related to it have been clarified in *A. thaliana*. Recently, homologs of *TTs* in *Brassica* have been comprehensively identified by Qu et al. [3], as well as the expression patterns in different yellow- and black-seeded *B. napus* inbred lines. In the present study, we compared



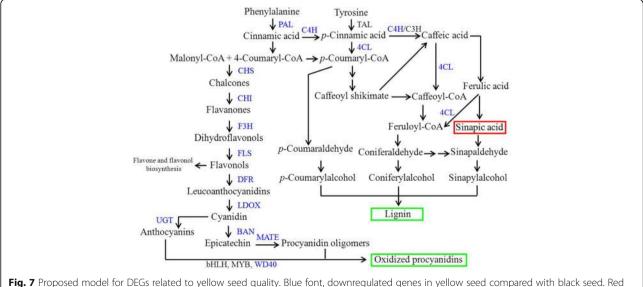
the expression pattern of genes related to flavonoid biosynthesis in developing seeds of B. napus cv. 'Yangyou 6' and yellow-seeded B. napus selected from somatic hybrids of B. napus-S. alba (Fig. 4). Four homologs of BnBAN (BnaA01g36200D, BnaA03g60670D, BnaC01g29820D and BnaC04g18950D) were downregulated at two to four consecutive developmental stages of yellow rapeseed compared with black rapeseed. Two homologs of BnDFR (BnaA09g15710D and BnaC09g17150D) were also expressed at lower levels in yellow seed, which encodes an enzyme catalyzing dihydroflavanones to leucoanthocyanidins that was then converted to anthocyanidin [52]. **BnLDOX** (BnaC01g14310D, BnaA03g45610D and BnaC07g37670D) was downregulated in yellow seed, which encodes leucoanthocyanidin reductase and catalyzes the formation of anthocyanidins. Genes encoding chalcone synthase (BnaA10g19670D, BnaC09g43250D,

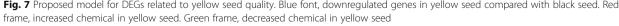
BnaC03g06120D, BnaC02g05070D and BnaC08g26020D) were also downregulated throughout yellow seed development, which catalyzes the first step of flavonoid biosynthesis. BnTT5 (BnaC08g26020D) was also downregulated in yellow seed compared with black seed at specific stages, encoding chalcone isomerase that is redundant and responsive to lights in Brassicaceae [53]. BnTT8 (BnaC09g24870D) and BnaA09g22810D) was also downregulated in yellow seed compared with black seed at specific stages, encoding the transcription factor bHLH as an important regulator throughout flavonoid biosynthesis. Another two bHLHs (BnaA01g17420D and BnaA01g24020D) were also downregulated, which have been proven to have functions in regulating jasmonate responses [54] and glucose homeostasis, and may affect ABA or salinity response in Arabidopsis [55]. In the present study, 21 CYPs were differentially expressed between yellow and black rapeseeds, although

they were not shown to have functions in the secondary metabolism of B. napus. Previously, CYP71/75/93/81 have been reported to have functions in the biosynthesis of anthocyanin pigments and condensed tannins [42]. Lam et al. found that CYP75B4 and CYP93G1 promote tricin accumulation in Arabidopsis and O. sativa, which functions in generating 3'-hydroxylated flavonoids and flavone aglycones [56, 57]. Recently, Lei et al. found that a group of CYPs, including members of CYP71/72/77/78/81/85/86/ 90/93, were differentially expressed in Dendrobium catenatum from different locations and with different outlooks, which might be related to flavonoid biosynthesis [58]. CYP86 has been reported to be involved in suberin monomer biosynthesis in Arabidopsis [59]. Expressional differences of five TCPs were also identified, which might be related to flavonoid biosynthesis since TCP3 could interact with R2R3-MYB [41]. In addition, TCP1 directly activates DWARF4 and promotes brassinosteroid biosynthesis [60].

Asides from flavonoids, lignin has been revealed to have a positive correlation with seed coat color [26]. We found that 4CL, C4H and PAL were downregulated in yellow rapeseed compared with black seed, which play important roles in the less lignin in yellow seed (Fig. 4). PAL is involved in the first step of the phenylalanine metabolic pathway by catalyzing phenylalanine into cinnamic acid. Cinnamic acid was then transformed into coumaric acid by C4H. 4CL plays an important role in several steps of lignin biosynthesis and participates in the formation of coumaroyl-CoA, caffeoyl-CoA and feruloyl-CoA. Previously, Jiang et al. (2013) and Wang et al. (2018) reported that sinapic acid was more accumulated in yellow seed than black seed [39, 61]. Down-regulation of 4CL in yellow rapeseed indicated that caffeic acid and ferulic acid might be less transformed into caffeoyl-CoA and feruloyl-CoA. Thus, sinapis acid as another downstream chemical of caffeic acid and ferulic acid was more accumulated (Fig. 7). All these compounds are necessary intermediates of lignin biosynthesis [5]. Hong et al. found that the expression of genes involved in lignin biosynthesis were slightly but not markedly changed in yellow-seeded NILs [50]. As to the other DEGs related to secondary metabolism, we found genes encoding S-adenosylmethionine synthetase 2 (BnaA09g00390D), peroxidase (BnaA02g14050D, BnaC06g32660D, BnaC06g21080D), branched-chain amino acid transaminase 1 (BnaA01 g36200D), methionine synthase 2 (BnaC03g33530D), aspartate aminotransferase 5 (BnaC01g06460D), phosphoglycerate kinase (BnaA07g34980D, BnaC02g46710D), lactate/malate dehydrogenase (BnaA09g16400D), glucose-1-phosphate adenylyltransferase family protein (BnaC04g33380D), beta gluco-19/28(BnaA05g04030D, BnaC01g43700D, sidase BnaC05g11190D, BnaA06g09700D), and pyruvate kinase (BnaA03g36910D) were downregulated in yellow rapeseed (Additional file 11: Figure S5). Peroxidase is one of three major enzymes involved in flavonoid oxidation through the associated reduction of hydrogen peroxide in the peroxidative cycle [62]. Hong et al. assigned peroxidase superfamily protein (e.g., peroxidase 4/7/25/52/53) to phenylpropanoid and flavonoid pathways, which were differentially expressed between yellow and black rapeseed NILs [50]. The DEGs related to flavonoid and lignin biosynthesis confirmed that they contribute to the different testa color.

Since the oil content of yellow seed was higher than that of black seed, we found 12 DEGs related to FA biosynthesis, metabolism, elongation and degradation





(Fig. 5). In addition, we found that BnaA09g51510D (encoding pyruvate dehydrogenase E1 alpha) was upregulated throughout yellow seed development compared with black seed (Additional file 11: Figure S5). Its homolog in A. thaliana is important for seed oil biosynthesis. Plastidic pyruvate kinase (PKP) provides most of the pyruvate for plastidic FA synthesis, and the mutation of *PKP* severely impairs seed storage lipid synthesis [63]. BnaA03g17570D (acetyl Coenzyme A carboxylase carboxyltransferase alpha subunit) was upregulated in yellow seed. Its homolog in A. thaliana has been confirmed to be a subunit of heteromeric acetyl-CoA carboxylase (ACCase), which catalyzes the carboxyltransferase (CT) reaction. ACCase is responsible for the first step of FA synthesis [64]. Additionally, homolog of UDP-glucose (UGP)(BnaA05g32480D), pyrophosphorylase а sucrose-regulated protein, was upregulated in yellow seed, which is required for fumonisin B1-induced cell death [65]. A homolog of beta glucosidase 25 (BnaC01g40610D) was also upregulated in yellow seed, which is involved in the carbohydrate metabolic process [66]. Upregulation of cytochrome B5 reductase 1 (CB5R1) (BnaC02g02110D) in yellow seed might also be related to the FA variation, since CB5R is a microsomal membrane-bound protein that functions as part of the microsomal electron transfer system in FA desaturation [67]. CB5R can interact with ankyrin repeat-containing protein 2A (AKR2A), which interacts with ascorbate peroxidase 3 (APX3). APX3 can target peroxisomes [68]. This might be related to the above-mentioned expressional changes in genes encoding peroxidases. The expression changes of these genes may be helpful in explaining the FA variation in yellow seed and its adaptability to environments after flavonoid reduction.

# Conclusions

In the present study, whole transcriptome gene expression was analyzed in developing seeds of yellow-seeded B. napus derived from hybrids of B. napus-S. alba, and black-seeded B. napus. We identified the DEGs with seed development, which might be related to the development and biosynthetic process. In addition, DEGs related to the quality difference between yellow and black rapeseed have been identified, which mainly participate in flavonoid biosynthesis, phenylpropanoid biosynthesis, phenylalanine metabolism, flavone and flavonol biosynthesis, fatty acid biosynthesis and metabolism. These down-regulated genes are helpful to explain the less pigmentation (e.g. CHS, CHI, F3H, FLS, DFR, LDOC, BAN) and lignin (PAL, C4H and 4CL), and higher oil content in yellow rapeseed compared with black seed (Fig. 7). Future functional analysis of these genes would contribute to the molecular dissection of yellow seed character in B. napus.

# Methods

# Plant material

Yellow-seeded B. napus (line W82) was preserved in our lab and was selected from back-crossing progenies of somatic hybrids of B. napus-S. alba. The black-seeded rapeseed (B. napus cv. 'Yangyou 6') was obtained from the Jiangsu Lixiahe Region Agricultural Research Institute, China [45]. Both rapeseed lines were grown in the experimental field of Yangzhou University, Yangzhou, China. In addition to the visible difference in seed coat color, the flavonoid content in yellow seed was lower than that in black seed, and the seed FA composition and content were different between yellow- and black-seeded B. napus. The oil content of W82 was 6% higher than that of Yangyou 6. Higher protein and sucrose contents, less dietary fiber and crude fiber, and fewer glucosinolates accumulated in the seed meal of yellow rapeseed compared with black rapeseed (Additional file 12: Table S7) [8, 39, 61, 69]. The developing seeds at 3 WAF, 4 WAF, 5 WAF, 6 WAF, and mature seeds were collected from three pods each of ten individual plants for comparative analysis. During the seed development, differentially accumulated pigments were visual since 5 WAF of yellow and black rapeseeds. Proanthocyanidins (PAs) were less accumulated throughout yellow seed development than black seed [39, 70].

RNA extraction, library construction, and RNA sequencing For each developmental stage of each rapeseed line, five RNA samples were separately isolated from polled seeds of ten plants using Trizol Reagent Solution (Invitrogen, USA). Each library was pooled by mixing equal quantities of five RNA samples. The RNA quality was validated using agarose gel electrophoresis, Nanodrop, Qubit, and Agilent 2100 to confirm the purity, concentration, and integrity, respectively. mRNA was purified using beads with Oligo (dT), and cDNA was then synthesized with random hexamers after fragmentation of mRNA. After purification of cDNA with AMPure XP beads, unique adaptors and indexes were ligated. Certain fragments were selected with beads and enriched using PCR amplification. Finally, ten cDNA libraries for five developmental stages (3 WAF, 4 WAF, 5 WAF, 6 WAF and mature seeds) of yellow- and black-seeded B. napus were normalized based on a Qubit assessment, and the insert size was validated by Agilent 2100. Then, the polled libraries were sequenced by the Illumina HiSeq™ 2000 platform.

### **Bioinformatics analysis**

After removing adaptor sequences and low-quality sequences, clean reads were mapped to the *B. napus* cv. Darmor-*bzh* genome (version 5) using TopHat2 [71, 72].

Based on the predicted gene models of Cufflinks, classification and statistics of alternative splicing were carried out using AS profile [73, 74]. Novel transcripts were predicted using Cufflinks to assemble the mapped reads on the genome and compare it with the known gene models [75]. The abundance of reads mapped to reference was estimated and normalized using Reads Per Kilo bases per Million reads (RPKM), and all the transcripts with RPKM value> 1 were used for further analysis. Differential gene expression analysis was performed using DESeq [76, 77], and DEGs between libraries were screened with a threshold of | (FoldChange)| > 1 and q value< 0.005. All the DEGs among different libraries were clustered base on the  $log_{10}(RPKM+1)$  and  $log_2(Fold Change)$ value. Gene ontology (GO) enrichment of the DEGs was performed using GOseq with a corrected *p*-value < 0.05 [78], and KEGG pathway enrichment of DEGs was performed using KOBAS 2.0 with a corrected *p*-value < 0.05 [79]. An overview of pathways related to these DEGs was predicted by MapMan (version 3.6.0) analysis [80].

#### cDNA synthesis and qRT-PCR validation

Subsamples of RNA-Seq were reverse transcribed into cDNA for real-time qPCR validation using the Revert Aid First Strand cDNA Synthesis Kit and SYBR Green Real-Time PCR Master Mixes (Thermo, USA). qRT-PCR was performed on a fluorescence quantitative system Mx3005P (Agilent, USA). Genes, primers and size of the amplicon are listed in Additional file 13: Table S8. *B. napus*  $\beta$ -actin (NCBI AF111812) was used as an endogenous control to generate the  $\triangle$ Ct for three technological replicates.

## Additional files

Additional file 1: Table S1. Summary of read mapping in RNA-Seq analysis of yellow and black rapeseeds. B3, B4, B5, B6 and BM indicate 3 ~ 6 WAF and mature black seed. Y3, Y4, Y5, Y6 and YM indicate 3 ~ 6 WAF and mature yellow seed. (XLSX 34577 kb)

Additional file 2: Table S2. DEGs identified with black or yellow seed development and between same developmental stages of two rapeseeds. (XLSX 11030 kb)

Additional file 3: Table S3. DEGs related to seed development. (XLSX 94 kb)

Additional file 4: Table S4. Up- and downregulated genes among all the developmental stages of yellow- and black-seeded *B. napus.* (XLSX 25 kb)

Additional file 5 Figure S1. The SOM clusters of DEGs from five developmental stages of two rapeseed lines. (XLSX 173 kb)

Additional file 6: Table S5. All the up- and down-DEGs with GO annotation. (XLSX 63 kb)

Additional file 7: Figure S2. KEGG enrichment of DEGs (Y3 vs. B3) in flavonoid and phenylpropanoid biosynthesis. (XLSX 24 kb)

Additional file 8: Figure S3. KEGG enrichment of up- and down-DEGs between yellow- and black-seeded *B. napus*. (XLS 31 kb)

Additional file 9: Table S6. KEGG pathways of DEGs between yellowand black-seeded *B. napus.* (PDF 519 kb)

Additional file 10: Figure S4. Overview of pathways related to the DEGs between yellow- and black-seeded *B. napus.* (PDF 981 kb)

Additional file 11: Figur S5. Heatmap of DEGs involved in secondary metabolism. (JPG 6164 kb)

Additional file 12: Table S7. Quality differences between yellow- and black-seeded *B. napus. (JPG 1896 kb)* 

Additional file 13: Table S8. Primers for qPCR validation of DEGs (JPG 3234 kb)

#### Abbreviations

4CL: 4-coumarate CoA ligase; ACCase: Acetyl-CoA carboxylase; AKR2A: Ankyrin repeat-containing protein 2A; APX3: Ascorbate peroxidase; C4H: Cinnamate 4-hydroxylase; CBSR1: Cytochrome B5 reductase 1; CT: Carboxyltransferase; CYP: Cytochrome P450; DEG: Differentially expressed gene; EBG: Early biosynthesis gene; FA: Fatty acid; GO: Gene ontology; GWAS: Genome wide association study; KEGG: Kyoto encyclopedia of genes and genomes; LBG: Late biosynthesis gene; LEA: Late embryogenesis abundant hydroxyproline; MBW: MYB-bHLH-WD40; NIL: Near-isogenic line; PA: Proanthocyanin; PAL: Phenylalanine ammonia-lyase; PKP: Pyruvate kinase; qRT-PCR: Quantitative real-time polymerase chain reaction; RPKM: Reads per kilo bases per million reads; SNP: Single nucleotide polymorphism; TT: Transparent testa; UGP: UDP-Glucose pyrophosphorylase; WAF: Weeks after flowering

# Acknowledgements

Not applicable.

#### Funding

This study was supported by the National Key Basic Research Program of China (2015CB150201), the National Natural Science Foundations (31330057, 31771825, 31401414), the National Key Research and Development Program of China (2016YED0101000, 2016YFD0102000), the Natural Science Foundation of Jiangsu Province (BK20180101, BK20140478), China Postdoctoral Science Foundation (2015 T80591, 2014 M561719), Jiangsu Postdoctoral Science Foundation (1401078B), the Postgraduate Research & Practice Innovation Program of Jiangsu Province (XKYCX17\_066), the Priority Academic Program Development of Jiangsu Higher Education Institutions, and Yangzhou University for Excellent Talent Support Program. The founders did not play any roles in the design, analysis, interpretation of this study or relevant data.

#### Availability of data and materials

All the data pertaining to the present study have been included in the tables and figures of the manuscript, and the authors are pleased to share all the data and plant materials upon reasonable request.

#### Authors' contributions

JJ performed the experiments, analyzed the RNA-seq data, and drafted the manuscript. SZ, YY, YW and LZ sampled the materials and performed qRT-PCR analysis. JB and YPW revised the manuscript. All the authors approved the final manuscript.

### Ethics approval and consent to participate

This study has not directly involved humans or animals. The yellow-seeded *B. napus*, which originated from somatic hybridization between *B. napus* and *S. alba*, was created and preserved by our group. The black-seeded *B. napus* was obtained from the Jiangsu Lixiahe Region Agricultural Research Institute, China. No specific permission was required for use of these materials for experimental purposes. The seedlings were grown in the experimental field of Yangzhou University, Yangzhou, China as per standard practices, and samples were harvested at the required time. We comply with the Convention on the Trade in Endangered Species of Wild Fauna and Flora.

#### Consent for publication

Not applicable.

#### **Competing interests**

The authors declare that they have no competing interests.

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Received: 1 November 2018 Accepted: 7 May 2019 Published online: 16 May 2019

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