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Deep sequencing of the transcriptome reveals distinct flavonoid metabolism features of black tartary buckwheat (*Fagopyrum tataricum* Garetn.)

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ABSTRACT

Black tartary buckwheat is recognized as 'black pearl' because of containg more rutin and other flavonoids as compared to yellow tartary buckwheat (traditional tartary buckwheat). Here, we show a genome-wide comparison of their transcriptomes by using an RNA-seq approach to elucidate the different molecular metabolism on the flowers from Black tartary buckwheat (HEIFENG No1) and yellow tartary buckwheat (XIQIAO No2). Over 48.4 million paired-end reads were assembled into 57,800 unigenes, of which about 57.9% (33, 472 unigenes) were annotated by BLAST searches in the NCBI non-redundant protein database. RPKM analysis showed that compared to YTB, the unigenes encoding phenylalanine ammonialyase (PAL), chalcone synthase (CHS) and chalcone isomerase (CHI) for early flavonoid synthesis and the unigene encoding quercetin 3-O-glucosyltransferase (UF3GT) for synthetizing rutin were at a higher level, but the unigene encoding Flavonol synthase (FLS) charging for kaempferol and quercetin synthesis at a lower level in BTB, which may be the reason for the higher content of rutin and the lower content of quercetin, the result obtained by HPLC, as confirmed by qRT-PCR analysis of these genes. The result will not only explain the molecular mechanism of flavonoid synthesis in balck tartary buckwheat, but also provide the basis for further genomics research on this species or its allies.

Contents

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1. Introduction

Buckwheat is a plant belonging to the genus *Fagopyrum*, which is a member of the eudicot family Polygonaceae comprising about 17 species (Ohnishi, 1991, 1998). One of the species, tartary buckwheat (*Fagopyrum tataricum* Garetn.), has gradually become a recognized model organism for research on rutin metabolism due to the abundance of rutin, which is 0.8–1.7% of the dry weight of the plant and 50–80% of total flavonoids content (Fabjan et al., 2003). Rutin, quercetin, isoquercetin and other flavonoids in tartary buckwheat have antidiabetic activity (Zhang et al., 2011; Yao et al., 2008), anti-inflammatory activity (Karki et al., 2013), anti-fatigue properties (Jin and Wei, 2011) and can be used to treat microangiopathy (Tsai et al., 2012; Ushida et al., 2008; Li et al., 2002) and prevent liver inflammatory injury (Lee et al., 2013). In addition, some proteins in buckwheat have anti-tumour activity (Guo et al., 2007, 2010; Ren et al., 2001, 2003). Tartary buckwheat improves cognition and memory function (Abbasi et al., 2013; Choi et al., 2013). Tartary buckwheat cookies, buckwheat sprout, buckwheat product and buckwheat bran extract lower plasma and lipid cholesterol levels (Wieslander et al., 2011; Liu et al., 2008; Tomotake et al., 2007, 2015; Meschini et al., 2015); therefore, tartary buckwheat has gradually been a focal point of research.

Black tartary buckwheat (BTB) is a new developed variety based on the traditional tartary buckwheat. It has dark black shell that that is different from the traditional tartary buckwheat the colour of which is yellowish white (yellow tartary buckwheat, YTB). The fruit from BTB is bigger, fuller and contains higher nutritional value than that from YTB, such as the rutin content of BTB being higher than that of YTB (Park et al., 2004), so black tartary buckwheat is known as “the black pearl” in Sichuan province of China. Because rutin content in the flower was highest in all tissues (Park et al., 2004), the flowers of BTB were used as our primary research material.

To better understand the distinct features of BTB, we used an RNA-seq approach to measure differences in the mRNA transcriptome, used high Performance Liquid Chromatography (HPLC) and the ultraviolet spectrophotometer to measure kaempferol, quercetin, rutin, myricetin and total flavonoids content difference of the flowers of YTB and BTB during the flowering stage prior to the subsequent seed stage. Combined with quantitative RT-PCR, various genes involved in flavonoid metabolism were shown to be differentially expressed, which is useful to understand BTB as an excellent medical and nutrient-rich crop.

2. Results and discussion

2.1. Flavonoids difference between BTB and YTB

To know more about flavonoid characters in BTB, we compared flavonoids content and total flavonoids content in BTB and YTB by using HPLC and ultrasonic methods. It is found that the valuable substance content such as total flavonoids and rutin in BTB are more than that in YTB (Table 1). For example, the average of the amount of total flavonoids from flowers in YTB and BTB was 49.07 μg/mg and 52.81 μg/mg (DW), which indicate that of BTB is 7.63% more than that in YTB (Fig. 11). Rutin content in YTB and BTB is about 35.93 μg/mg and 38.80 μg/mg, accounting for 73.2% and 73.5% in total flavnoids. The rutin content in BTB is 8.0% more than that in YTB. But as a precursor in rutin biosynthesis pathway, quercetin content in YTB and BTB is 3.06 μg/mg and 6.49 μg/mg, which indicate that quercetin content of BTB is 52.9% less than that of YTB. The kaempferol content in BTB is also less than that in YTB, but the myricetin in BTB is the same as that in YTB.

2.2. De novo assembly of sequences and construction of unigene library

In the present study, in order to ensure the inclusion of the maximum number of genes in the transcriptome, we performed the Illumina GAIIX platform run on each of two cDNA libraried constructed from flowers in *Fagopyrum tataricum* cultivars with contrasting seed *F. tataricum* cultivar XIQIAO No. 2 (YTB, yellow tartary buckwheat) and *F. tataricum* cultivar HEIFENG No. 1 (BTB, black tartary buckwheat) (Fig. 1). The total number of reads obtained was 21.6 million and 26.8 million with a mean raw read length of 101 bp in YTB and BTB, which corresponded to 4,361,917,824 bp and 5,417,027,031 bp, respectively (Table 2). After adapter trimming, B-trimming and low-quality end-trimming, the high quality reads were used for de novo assembly to generate 756,343 contigs and 912,830 contigs respectively. Due to the non-availability of a reference genome sequence, the assembly of the high quality reads was done using Trinity (v3.3.5), which resulted in 44,043 transcripts and 46,544 transcripts with an average read length of 908.14 bp and 896.60 bp in the two kinds of flowers, indicating an increased coverage and depth of sequencing by generating longer fragment lengths (Table 2). Finally, 82,128 unigenes were assembled in XIQIAO No. 2 unigene library and 87,062 unigenes were assembled in HEIFENG No. 1 unigene library and 57,800 unigenes were assembled in both types in *F. tataricum*. With an increase in the length, the number of all unigenes decreased (Fig. 2).

In 2011, Logacheva et al. performed flower transcriptome sequencing by 454 sequencing. Recently, Haifeng et al. performed root and shoot transcriptome sequencing by Illnima sequencing. In comparison with the results, we produced more assembled unigenes (57,800 vs 39,815 vs 25,401), although the average length of unigenes is not the longest in the data (954.83 vs 1184 vs703) (Table 2). Therefore, the assembled unigene database in our study should provide a useful resource for future research on *F. tataricum*, especially on the flower.

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Table 1
Flavonoid content (mg/500 mg fresh weight FW.%) in different species of *F. tataricum*.

Organ	Total flavonoid content	Rutin	Kaempferol	Quercetin	Myricetin
BTB	52.81 ± 1.56	38.80 ± 0.017	0.0556 ± 0.000	3.06 ± 0.000	0.43 ± 0.002
YTB	49.07 ± 0.91	35.93 ± 0.795	0.0934 ± 0.002	6.49 ± 0.003	0.40 ± 0.001

Results are expressed as Mean ± SD (n ≥ 2).

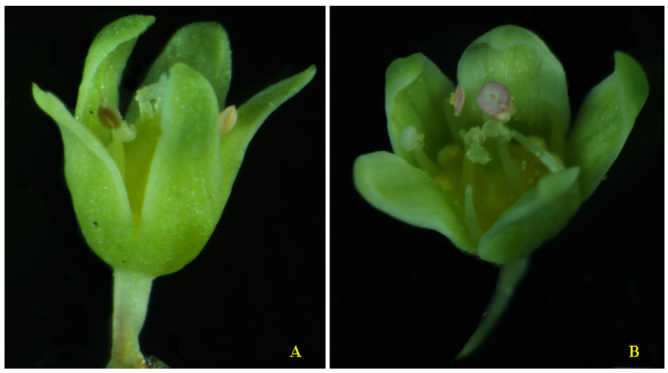

Fig. 1. Images of the flowers of the tartary buckwheat accessions used. (A) A flower from black tartary buckwheat(BTB); (B) A flower from yellow tartary buckwheat (YTB).

2.3. Gene and pathway annotation and analysis

The sequence similarity search was done by subjecting the unigenes to BLAST X analysis (e-value ≤ 1e-5) against the NR, NT, TrEMBL, SwissProt, COG, GO and KEGG databases. From a total of 57,800 unigenes, 33,472 (57.91%) sequences were annotated by being aligned to the NR database (S1 Table); 26,250 (45.42%) hits had similarity with the NT database (S2 Table); 33,526 (58.00%) were matched to the TrEMBL database (S3 Table); 27,346 (47.31%) had similarity with the Swiss-Prot database (S4 Table); 12,099 (20.93%) were matched to the COG database(S5 Table); 28,762 (49.76%) unigenes showed a significant similarity to the sequences available in the GO database (S6 Table); 8305 (14.37%) were matched to 158 pathways in the KEGG database (S7 Table, Table 3). 23,336 unigenes did not match any of the databases; this could be due to the presence of novel unigenes, the small size of the sequences or errors in sequencing (Table 4).

Table 2
Statistics of *Fagopyrum tataricum* transcriptome assembly.

Assembly quality parameters		
Type of tartary buckwheat	YTB	BTB
Number of raw reads generated	21,596,099	26,819,715
Total nucleotides	4,361,917,824	5,417,027,031
Number of contigs generated	756,343	912,830
Median contig length	107	99
Total contigs length	80,980,571	89,952,996
0 < contigs ≦ 300 b	715,754	870,215
300 < contigs ≦ 500 b	14,061	15,118
500 < contigs ≦ 1000 b	11,781	12,334
1000 < contigs ≦ 2000 b	9915	10,176
contigs ≧ 2000 b	4832	4987
N50	341	265
Number of transcripts generated	**44,043**	**46,544**
Median transcript length	908.14	896.60
Total transcripts length	39,997,323	41,731,274
200 < transcripts ≦ 300 b	10,938	11,898
300 < transcripts ≦ 500 b	10,627	11,386
500 < transcripts ≦ 1000 b	8828	9240
1000 < transcripts ≦ 2000 b	8701	8856
transcripts ≧ 2000 b	4949	5164
N50	1554	1557
Number of unigenes generated	**82,128**	**87,062**
Median unigene length	1202.89	1223.46
Total unigenes length	98,791,341	106,517,161
200 < unigenes ≦ 300 b	12,578	13,607
300 < unigenes ≦ 500 b	13,793	14,596
500 < unigenes ≦ 1000 b	17,481	18,183
1000 < unigenes ≦ 2000 b	23,396	24,303
unigenes ≧ 2000 b	14,880	16,373
N50	1833	1876
Number of unigenes generated	**57,800**	
Median contig length	954.83	
Total unigenes length	55,189,159	
200 < unigenes ≦ 300 b	14,431	
300 < unigenes ≦ 500 b	13,219	
500 < unigenes ≦ 1000 b	11,313	
1000 < unigenes ≦ 2000 b	11,370	
unigenes ≧ 2000 b	7467	
N50	1676	

2.4. Gene ontology (GO)

Blast2GO was used to classify the transcripts into different plant gene ontology categories such as molecular functions, biological processed and cellular components. 57,800 unigenes were selected for GO annotation (Fig. 3).

Among the biological processes category, cellular process were the main group, representing the maximum number of unigenes (23,286, 40.29%), which were followed by metabolic processes (22,065, 38.17%), response to stimulus (16,592, 28.71%) and biological regulation (15,561, 26.92%), followed by developmental processes (12,776, 22.10%), cellular component organization or biogenesis (11,900, 20.59%), localization (10,329, 17.87%) and multicellular organismal processes (10,186, 17.62%).

The molecular function category,in decreasing order of abundance, included binding (18,172, 31.44%), catalytic activity (15,345, 26.55%), transporter activity (2,464, 4.26%), nucleic acid binding transcription factor activity (1,669, 2.89%), structural molecule activity (1032 1.79%), molecular transducer activity (921, 1.59%) and enzyme regulator activity (722, 1.25%).

The cellular responses category was represented by a large number of transcripts coding for proteins which belong to cell parts (25,173, 43.55%), cells (24,863, 43.02%), organelles (22,869, 39.57%), membranes (13, 252, 22.93%), organelle parts (11,271, 19.5%), macromolecular complexes (5,587, 9.67%), membrane parts (5,178, 8.90%), cell junctions (3,541, 6.13%), extracellular regions (3,313, 5.73%) and membrane-enclosed lumens (1,849, 3.20%).

2.5. COG classification

Eukaryotic Orthologous Groups is another form of COG (Clusters of Orthologous Groups) which is unique to eukaryotes (Tatusov et al., 2003). It aids in identifying orthologous proteins and representing the phyletic classification of proteins coded in the whole genome of almost 21 organisms including bacteria, algae and eukaryotes. The unigenes obtained in our study were compared with the COG database and classified into 24 categories. The majority of unigenes belonged to general function prediction (3,338, 5.78%), which was followed by transcription (1,641, 2.84%), replication, recombination and repair (1624, 2.81%) and signal transduction mechanisms (1,470, 2.54%) (Fig. 4). None of the unigenes belonged to extracellular structures.

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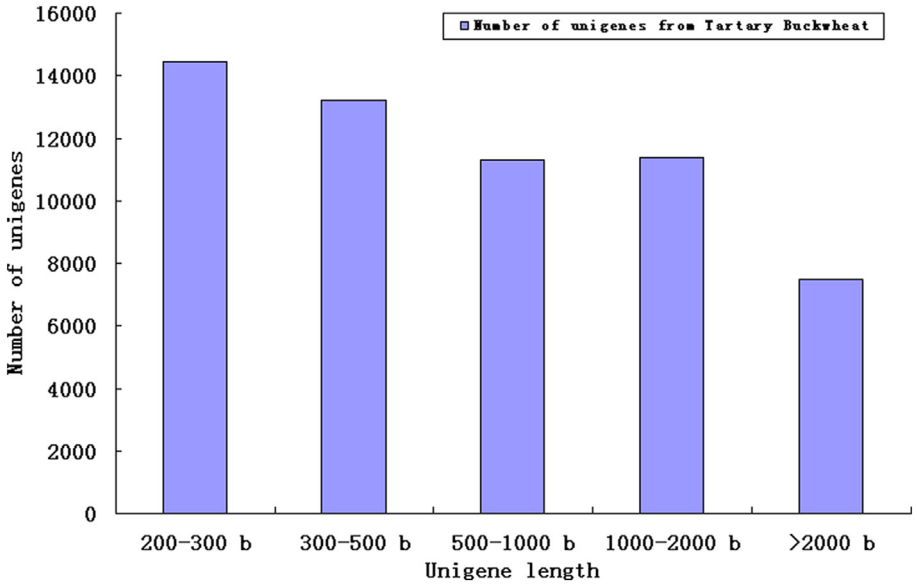

Fig. 2. Number of unigenes versus unigene length. Size distribution of unigenes based on the number of unigenes and unigene length.

Table 3
Statistics of *Fagopyrum tataricum* transcriptome annotation.

Anno_Database	Number annotated	Number of 300 bp–1000 bp	Number of >1000 bp	Percentage
nr_Annotation	33472	11848	18002	0.57910035
nt_Annotation	26250	8118	15706	0.45415225
TrEMBL_Annotation	33526	11875	18009	0.5800346
Swissprot_Annotation	27346	8966	15634	0.47311419
COG_Annotation	12099	3329	7741	0.20932526
GO_Annotation	28762	9656	16131	0.49761246
KEGG_Annotation	8305	2868	4396	0.14368512
All_Annotated	34464	12359	18079	0.59626298

Table 4
Comparison of our sequencing data with other sequencing data.

	Our data	Logacheva's data	Haifeng's data
Technology	Illumina sequencing	454 sequencing	Illumina sequencing
Organ	flower	flower	Root and shoot
No. of reads	48,415,814	229,031	267, 438, 632
Average length of reads	202	341	100
Total nucleotides	9.779 billion	0.078 billion	26.7 billion
No. of assembled unigenes	57, 800	25, 401	39,815
Average length of unigenes	954.83	703	1, 184

2.6. KEGG classification

The 8305 unigenes obtained in our study were compared with 158 KEGG pathways in the KEGG database. The majority of unigenes belonged to ribosome pathways (ko03010), including 539 unigenes, which was followed by plant hormone signal transduction (ko04075) (321), oxidative phosphorylation (ko00190) (303), protein processing in the endoplasmic reticulum (ko04141) (297) and glycolysis/gluconeogenesis (ko04075) (249) (Fig. 5).

2.7. Nucleotide content analysis and identification of SSRs

The GC content is an important criterion for establishing the phylogenetic and evolutionary relationships among various species, and its percentage varies among different organisms, ranging from 20% to 72%. The GC contents observed in BTB and YTB were around 45.97% and 46.20%, respectively (Table 5). Molecular markers play an important role in studies related to gene mapping and marker-assisted molecular breeding to improve plant varieties with desired traits (Yechezkel and David, 2006). Various molecular markers used for studying these variations include RFLP, RAPD, SNPs and SSRs. SSRs are microsatellites with 2–6 nucleotide tandem repeats which are distributed randomly throughout the genome of all eukaryotes (Yechezkel and David, 2006). Out of 18,837 unigenes that were examined, a total of 3779 SSRs were identified in *Fagopurum tataricum*. The statistical analysis of the identified SSRs is presented in Table 6. The number of mono, di, tri tetra and penta repeats was 2,098, 558, 1,085, 33 and 5, respectively(Fig. 6). The presence of more than one SSR was observed in 439 sequences.

2.8. Transcripts differentially expressed between BTB and YTB

To investigate the expression level of unigenes in BTB and YTB,

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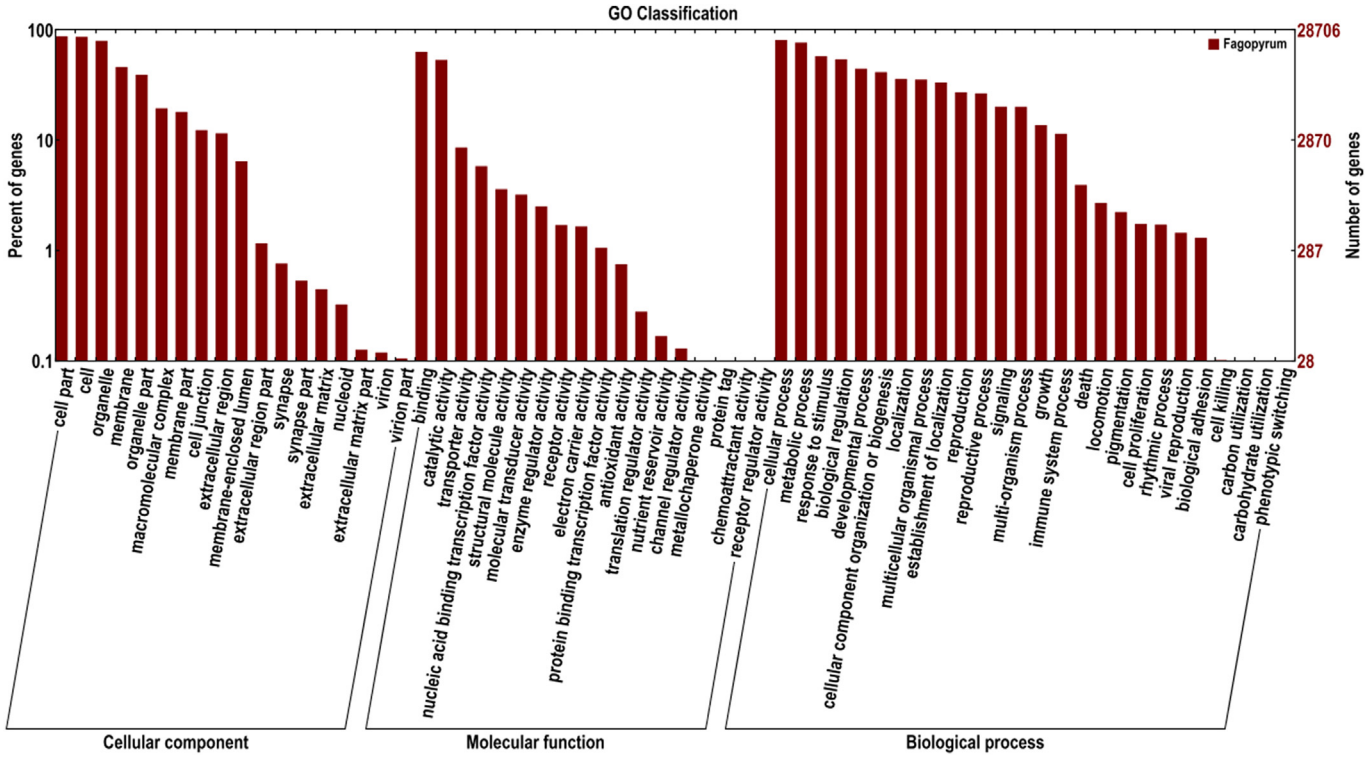

Fig. 3. GO classification. Gene ontology distribution of the unigenes into cellular components, molecular functions and biological processes. The number of unigenes encoded for each category is indicated.

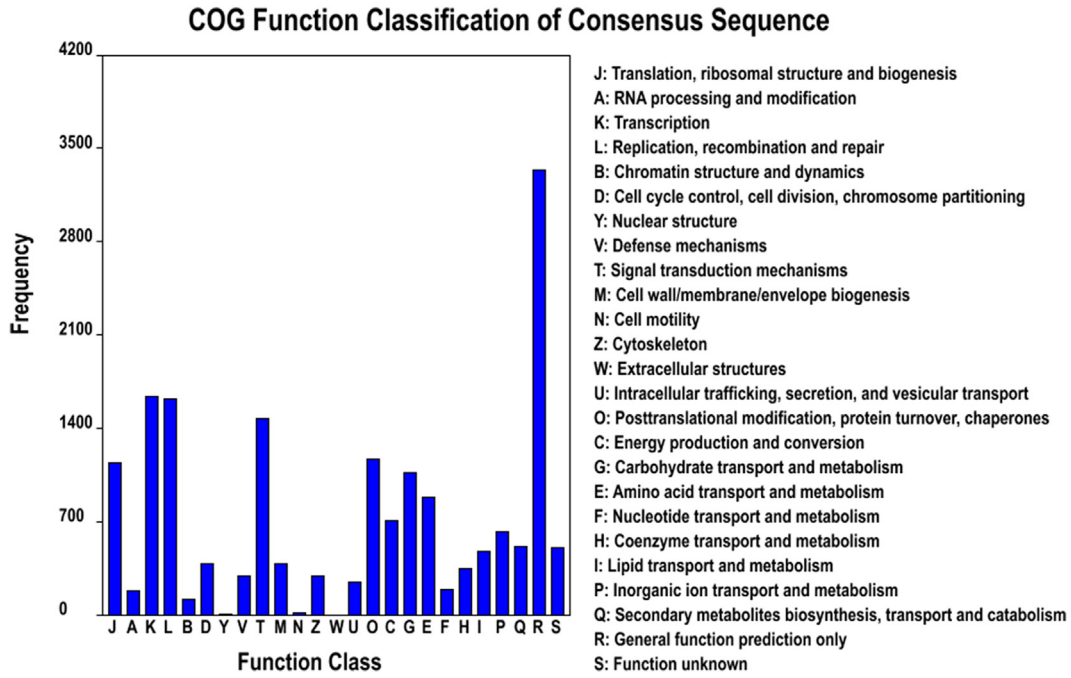

Fig. 4. KOG classification. Comparison of unigenes within the KOG database.

the number of clean reads was compared between the libraries for each of 57,800 unigenes through Reads Per Kilobase of transcript per Million (RPKM) analysis (Grabherr et al., 2011). Of the 824 unigenes found to be differentially expressed by more than 2-fold between YTB and BTB, 191 unigenes were upregulated while 633 were downregulated in the BTB as compared to the YTB. Clustering algorithms and Treeview were used to analyze the expression profiles of these genes (Fig. 7). In the 683 annotated unigenes, 179 unigenes were upregulated while 504 were downregulated by more than 2-fold (S8 Table). Among these unigenes, 83 (12.2%) unigenes showed more than a 16-fold change in expression level and 374 (54.8%) unigenes showed a 2~4-fold change. The fold

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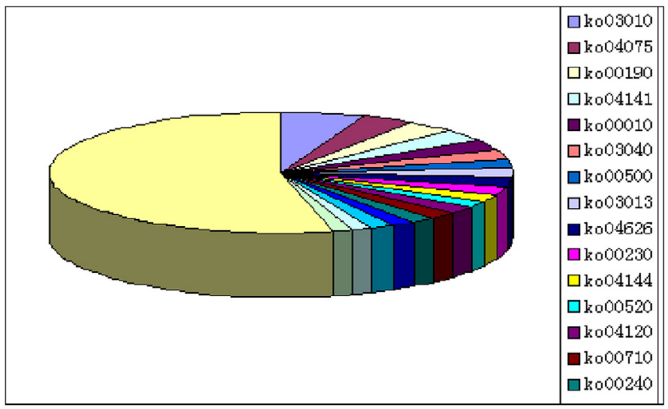

Fig. 5. KeGG classification. Kyoto Encyclopedia of Genes and Genomes distribution of the unigenes. The number of unigenes encoded for each path is indicated.

change distribution of unigenes differentially expressed between tartary buckwheats is shown in Fig. 8. Annotation of differentially expressed unigenes revealed that 683 unigenes belonged to 51 GO groups (Fig. 9) and 284 unigenes belonged to 25 functional categories (Fig. 10).

2.9. Analysis of the reason of total flavonoids and flavonol difference

In 57, 800 unigenes, 252 unigenes were involved in flavonoid biosynthesis, 464 unigenes were involved in positive regulation of flavonoid biosynthesis and 73 unigenes were involved in flavonol biosynthesis(S9 Table). In the unigenes, some key unigenes involved in flavonoid metabolism were differentially expressed. The first key enzyme of flavonoid biosynthesis is phenylalanine ammonialyase (PAL, EC 4.3.1.24), which catalyses a reaction converting L-phenylalanine to ammonia and trans-cinnamic acid and is the first and committed step in the phenyl propanoid pathway. It is therefore involved in the biosynthesis of the polyphenol compounds such as flavonoids, phenylpropanoids, and lignin in plants (Fritz et al., 1976; Tanaka et al., 1989). Many experiments have shown that the expression of PAL is related to the expression of total flavonoids (Hu et al., 2009). The second key enzyme of flavonoid biosynthesis is chalcone synthase (CHS, EC 2.3.1.74), which can use 4-coumaroyl-CoA and malonyl-CoA to produce CoA, naringenin chalcone, and CO_2 and act as a central hub for the enzymes involved in the flavonoid pathway (Crosby et al., 2011). CHS controls the speed of total flavonoids production by interacting with chalcone isomerase (Jez et al., 2000). Chalcone isomerase (CHI, EC 5.5.1.6) is also the master of the conversion efficiency of proflavonoids (Muir et al., 2001), which catalyses a reaction converting chalcone to flavanone, which can generate of flavanones, flavones, flavonols, anthocyanidins, isoflavanone, isoflavone and isoflavonol by the flavonoid branched metabolic pathways, respectively (Jez et al., 2000; Clain et al., 1997). According to S10 Table and Fig. 12, the expression of all unigenes of PAL, CHS and CHI in BTB is 22%, 14% and 16% more than that in YTB, which illustrates the reason that total flavonoids content from flowers in BTB is 7.6% more than that in YTB (Table 1, Fig. 11). Flavonol synthase (FLS, EC 1.14.11.23) is the enzyme that catalyses dihydroflavonol to produce the flavonol (Gert and Stefan, 2001), such as catalysing dihydrokaempferol to produce kaempferol, catalysing dihydroquercetin to produce quercetin and catalysing dihydromyricetin to produce myricetin (Fig. 13), so the expression of FLS is related to the content and the kind of the flavonol in *Petunia hybrida* (Holton et al., 1993). The sum of all FLS unigene RPKM values in BTB (556.97) was less than that in YTB (622.59), in which ten unigenes for FLS were upregulated and three unigenes for FLS were downregulated in BYB as compared to YTB (Fig. 13; S10 Table). The content of kaempferol and quercetin in BTB is about half that in YTB in Table 1, so it is supposed that some unigenes from Fagopyrum_T1_Unigene_BMK.35714, CL15183Contig1 and CL16015Contig1 control the two metabolic pathways that have been confirmed (Sun, 2005). UFGT (UDP-glucose: Quercetin 3-O-glucosyltransferase) is responsible for the glucosylation of quercetin to produce rutin. The sum of 22 UFGT unigene RPKM values in BTB (683.26) was more than that in YTB (614.02), which explains the rutin content in BTB is more than that in YTB (Table 1, Fig. 11), although its precurcor quercetin content in BTB is less than that in YTB (Table 1, Fig. 11).

Table 5
Number of transcripts involved in the flavonoid pathway.

Sample ID	Read Sum	Base Sum	GC (%)	N (%)	Q20%	Cycle Q20%
BTB	26819715	5417027031	45.97	0.00	97.72	100.00
YTB	21596099	4361917824	46.20	0.00	97.69	100.00

Table 6
Statistics of SSRs identified in the *Fagopyrum tataricum* transcriptome.

1	Total number of sequences examined	18,837
2	Total size of examined sequences (bp)	38,535,830
3	Total number of identified SSRs	3779
4	Number of SSR-containing sequences	3264
5	Number of sequences containing more than oneSSR	439
6	Number of SSRs present in compound formation	113

2.10. Real-time PCR analysis of the genes involved in the flavonoid biosynthesis pathway

To confirm the difference in expression level between the accessions found in RPKM analysis, five unigenes for flavonoid biosynthesis were chosen for qRT-PCR analysis (http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0071110Fig. 13, S10 Table). These Fagopyrum_T2_Unigene_BMK.22919 (PAL), Fagopyrum_T1_Unigene_BMK.36127 (F3′H), CL16091Contig1 (DFR), and CL9103Contig1 (UFGT) which were up-regulated in BTB, Fagopyrum_T1_Unigene_BMK.35714 (FLS) down-regulated. qRT-PCR data confirmed expression pattern of these unigenes by RPKM analysis (Fig. 14, S10 Table).

3. Conclusion

In this study, we have described a genome-wide comparison between the tartary buckwheat transcriptomes derived from one source of YTB and two sources of BTB. We have identified various genes that were differentially expressed in both types, which are potentially associated with the morphological and functional features of BTB. We have also identified various genes with well-characterised roles in flavonoid metabolism, which may be responsible for the higher nutritional value of BTB compared to YTB. These results highlight the valuable characteristics of black tartary buckwheat.

4. Materials and methods

4.1. Plant materials

In 2013, two types of tartary buckwheat seeds (YTB, yellow tartary buckwheat (XIQIAO No2); BTB, black tartary buckwheat

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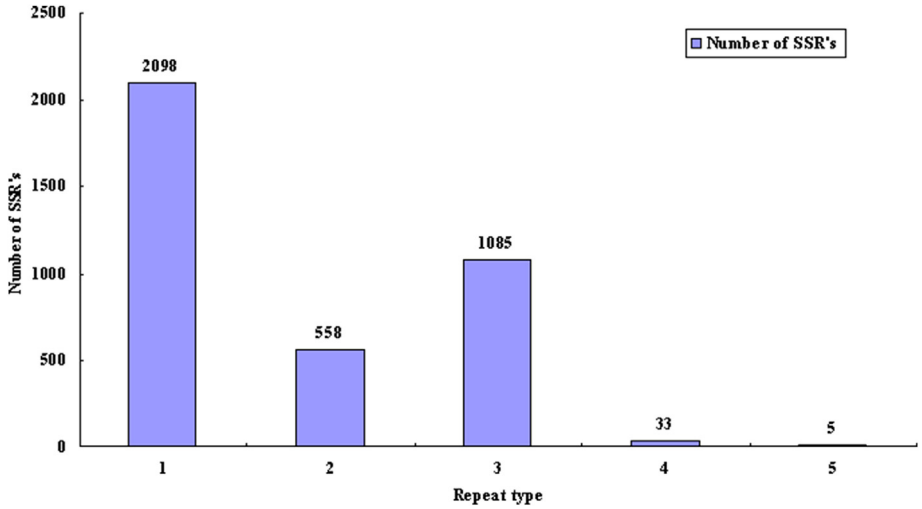

Fig. 6. Simple sequence repeats. Distribution of SSRs into di, tri, tetra, penta and hexa repeat types.

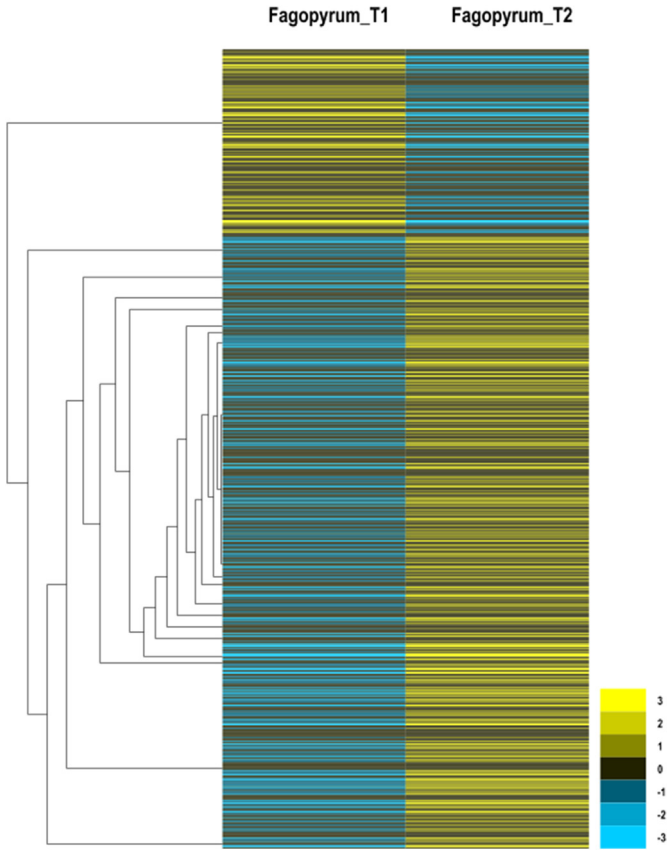

Fig. 7. Heat map of clustering of 824 genes expressed in YTB and BTB. Clustering was done using Cluster 3.0 software. The colour in the map display the relative values of all tiles within two strains of tartary buckwheat, blue indicates the lowest expression, black indicates intermediate expression, and yellow indicates the highest expression. The numerical values give the actual on a log 2 scale, which are associated with each colour. The colour scale bar is shown at the top right comer of the figure.

(HEIFENG No1)) were grown under field conditions on a farm at Baoxing, Ya'an, Sichuan, China on April 13. The flowers from two buckwheats were collected during the flowering stage, on June 15 and some flowers were treated by Sample Protector for RNA/DNA (Takara, China) and stored at -80 °C for mRNA extraction.

4.2. Determination of total flavonoids

Some flowers of two kinds of tartary buckwheats (500 mg each) were homogenised in 5 mL of 70% methanol containing 10% phosphoric acid (final, 0.1%) with a hand homogeniser. Flavonoids were extracted using an ultrasonic method. After cooling, the test tubes were centrifuged at 8000 × g for 10 min. The obtained supernatants were stored at −20 °C. Extracts from *F. tataricum* were analyzed with a spectrophotometer according to a previous report with a little modification (Tomotake et al., 2007). The reaction process was: 1 mL diluted extracts containing flavonoids, 0.3 mL of 5% (W/W) $NaNO_2$, and 4 mL of 70% methanol (V/V) were mixed for 6 min, and then 0.3 mL of 10% $Al(NO_2)_3$ (W/W) was added and mixed. Six minutes later, 4 mL of 1% NaOH (W/W) was added. The solution was diluted to 10 mL with 70% (V/V) methanol for measurement. After 15 min, the absorbance of the solution was measured at 500 nm with a UNICO WFJ2000 spectrophotometer (Unico, China). All samples were measured in triplicate.

4.3. Analysis of keampferol, quercetin, rutin and myritin content by HPLC

Two types of flowers were frozen in liquid nitrogen, ground to a fine powder using a mortar and pestle, and then extracted with 10 mL of 70% ethanol for 1 h at 60 °C. After centrifugation, the supernatant was filtered through a 0.45-μm poly filter (Acrodisc Syringe Filters, Pall, Port Washington, NY) and analyzed by HPLC. The analysis was performed with a C18 column (250 mm × 4.6 mm, 5 μm; RStech, Daejon, Korea) at 30 °C. The mobile phase was a gradient mixture of 0.2% acetic acid—water (A) and methanol (B). The solvent program was used as follows: 0 min solvent B 10%, 10 min solvent B 20%, 15 min solvent B 20%, 20 min solvent B 25%, 25 min solvent B 25%, 50 min solvent B 60%, 50.1 min solvent B 10%, and then constant solvent B 10% for 10 min (total, 60 min). The standard mixture of rutin, quercetin, kaempferol and myricetin were injected with a concentration range of 0.8–4.0 g/mL. The flow rate was maintained at 1.0 mL min^{-1}, the injection volume was 20 μL, and the detection wavelength was 280 nm. The concentration of flavones in the samples was calculated using the standard curve.

4.4. Total mRNA extraction

Total mRNA from the flowers of two kinds of tartary buckwheat

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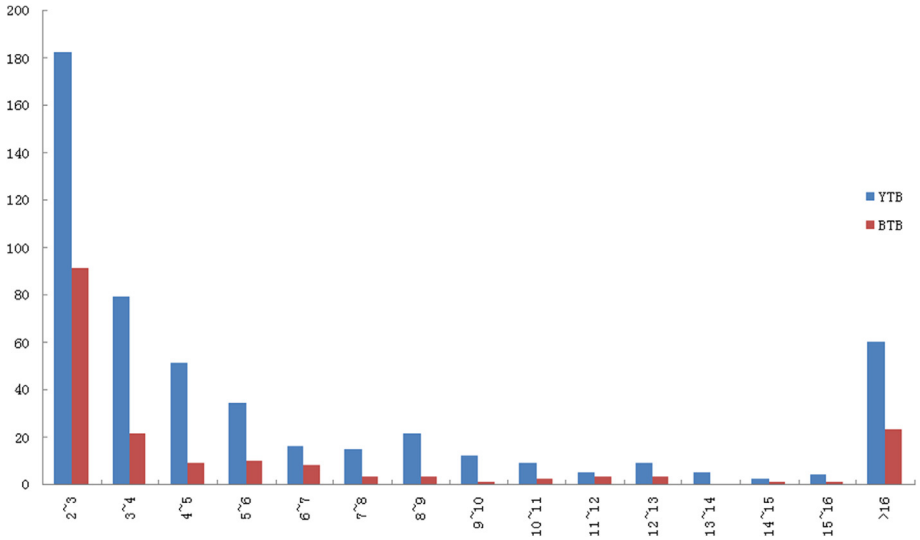

Fig. 8. The fold change distribution of differentially expressed genes between YTB and BTB.

was extracted using the RNAout kit (Tiandz, China) according to the manufacturer's procedure respectively. The purity and concentration of the isolated RNA were determined using the AgilentG2939A and Agilent RNA 6000 Nano LabChip Kit (Agilent, CA, USA). Two types of samples were obtained, with 260/280 values of 2.2 and 2.19 and RNA integrity numbers (RIN) of 8.6 and 8.5, respectively. These two kinds of RNA samples were analyzed and pooled to prepare an equimolar concentration of total RNA and were used for

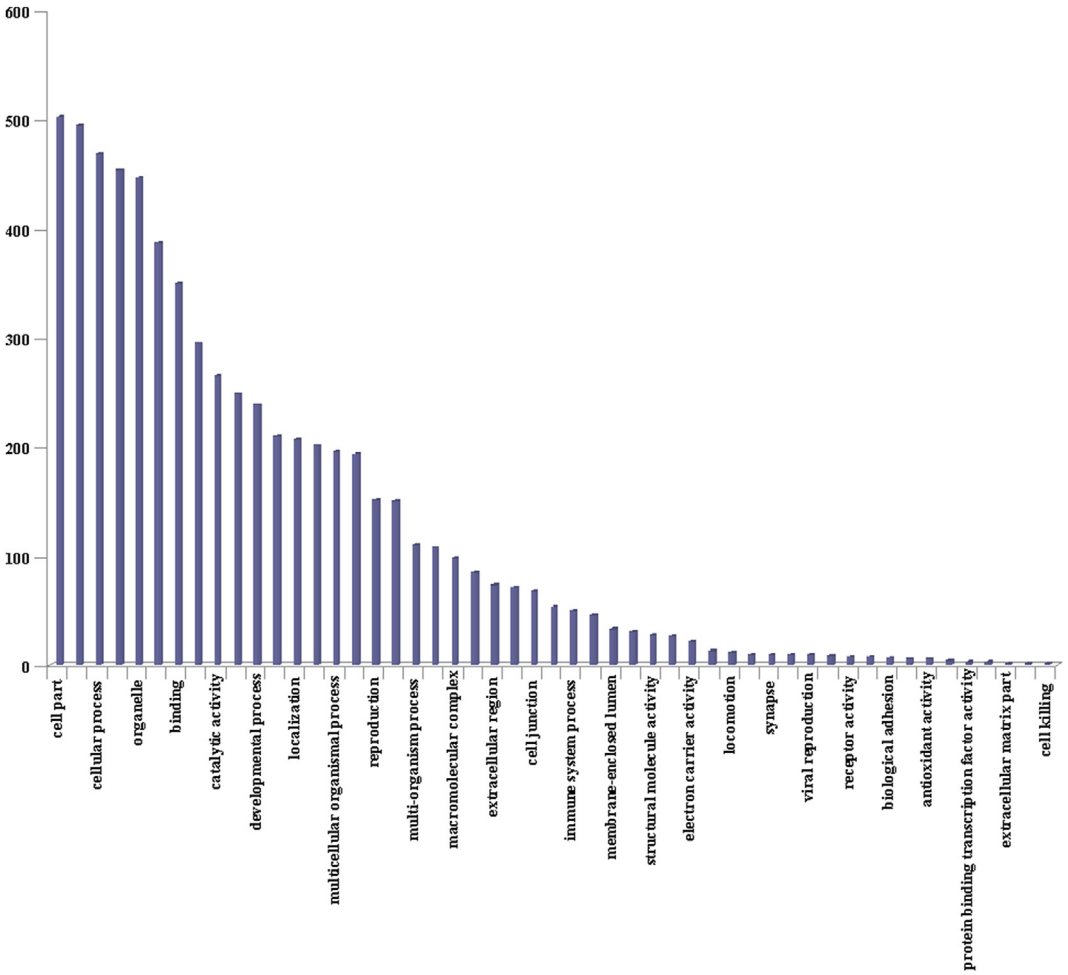

Fig. 9. Functional categories of unigenes differentially expressed between YTB and BTB.

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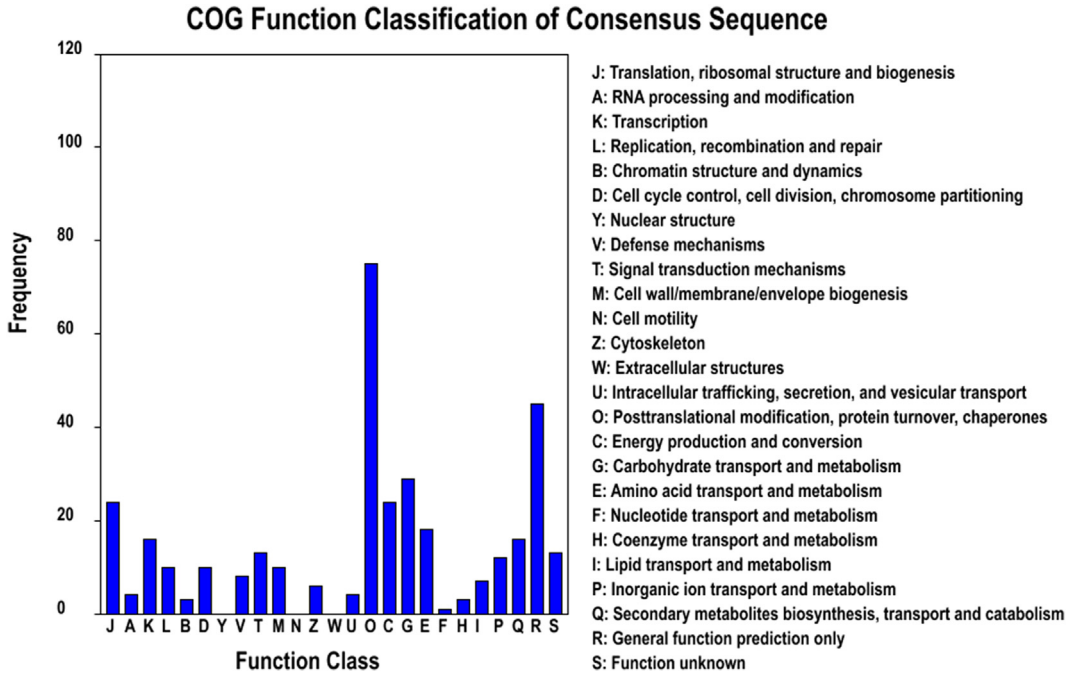

Fig. 10. COG function classification of DGE.

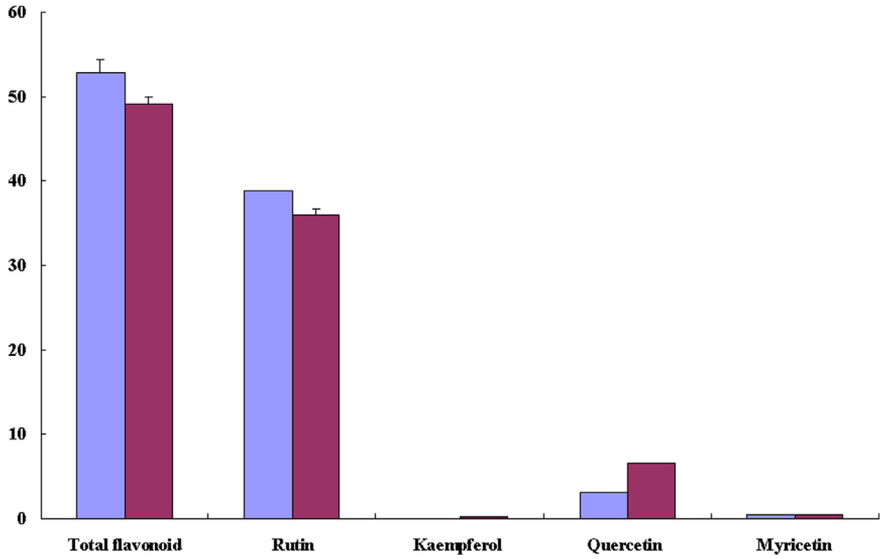

Fig. 11. The content of total flavonoid, rutin, kaempferol, quercetin and mycetin in flowers from YTB and BTB.

cDNA library construction respectively.

4.5. Transcriptome library construction and Illumina sequencing

The transcriptome library for Illumina sequencing was constructed according to the Illumina TruSeq RNA library protocol outlined in the TruSeq RNA Sample Preparation Guide respectively. To minimize the effect of transcriptome unevenness among individuals, equal quantities (5 μg) of total RNA isolated from YTB and BTB were pooled in 50 μl of RNase-free water and subjected to isolate poly(A) mRNA with poly-T oligo-attached magnetic beads (Invitrogen). Following purification, the mRNA was fragmented into small pieces using divalent cations at an elevated temperature. Then, the cleaved RNA fragments were reverse-transcribed to create the final cDNA library in accordance with the protocol for the mRNA-Seq sample preparation kit (Illumina, San Diego, USA). The average insert size for the paired-end libraries was 250 ± 50 bp. We then performed paired-end sequencing (101 bp) on an Illumina HiSeq™ 2000 apparatus (Illumina, USA) following the vendor's recommended protocol.

4.6. Sequence assembly and data analysis

The raw data from the images acquired after sequencing were transformed by base calling into raw reads and stored in FASTQ format. FASTQ reads were subjected to quality check using SeqQC V2.1 (Genotypic Proprietary Tool). The adaptor sequences, homopolymers and low quality bases were trimmed/filtered from the

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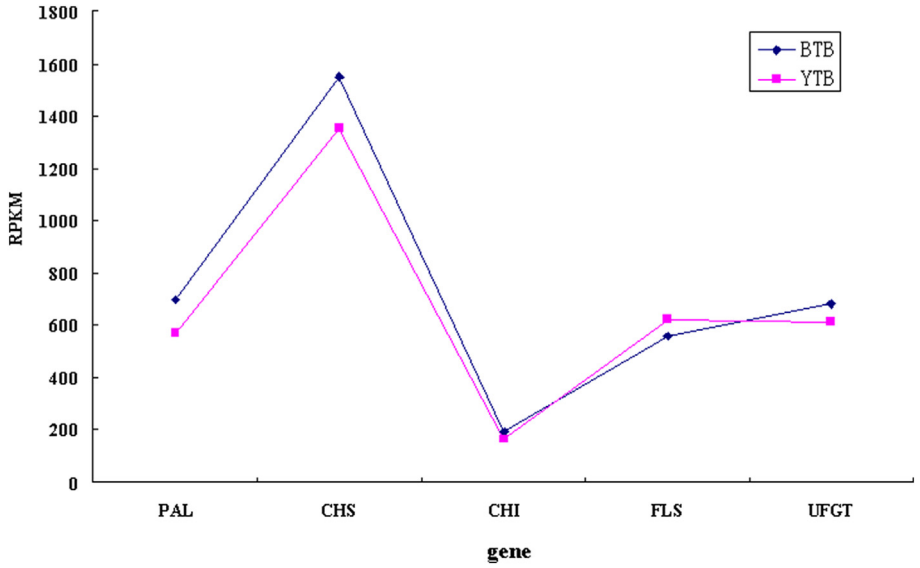

Fig. 12. RPKM of PAL, CHS, CHI, FLS and UFGT in flowers from YTB and BTB. Abbreviations: CHI, chalcone isomerase; CHS, chalcone synthase; FLS, flavonol synthase; PAL, phenylalanine ammonia-lyase; UFGT, UDP-glucose: Quercetin 3-O-glucosyltransferase.

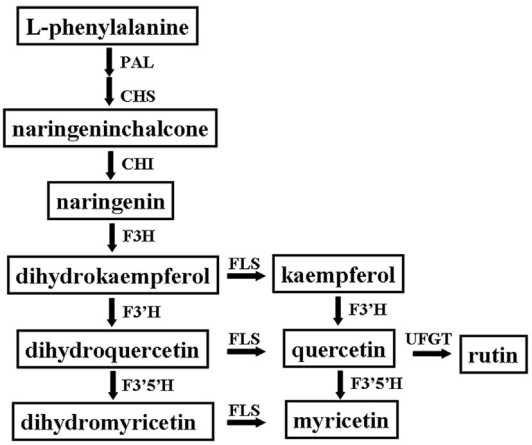

Fig. 13. Schematic of the flavonol pathway. Abbreviations: CHI, chalcone isomerase; CHS, chalcone synthase; FLS, flavonol synthase; F3′H, flavonoid 3′ hydroxylase; PAL, phenylalanine ammonia-lyase; F3′5′H, 3′5′ hydroxylase; F3H, flavanone 3-hydroxylase; UFGT, UDP-glucose: Quercetin 3-O-glucosyltransferase.

raw FASTQ data using the Custom Perl script to generate contigs database (Ravi and Mukash, 2012). Filtered contigs were de novo assembled using Trinity (Grabherr et al., 2011) (v3.3.5 with parameters: K-mer = 25, overlap = K-mer-1, group_pairs_distance = 500) to produce transcripts database. After de novo assembly, the transcripts from YTB, the transcripts from BTB and all transcripts from both types were clustered using BLAT (http://genome.ucsc.edu/cgi-bin/hgBlat) (parameters: tileSize = 8, -stepSize = 5) to produce three unigene libraries including the HEIFENG No. 1 unigene library, the XIQIAO No. 2 library and all unigene library having all unigenes by selecting the longest sequence in each cluster.

All unigenes in three libraries were annotated by Getorf (Mortazavi et al., 2008) to look for open reading frame (ORFs) in the database and were annotated using BLAST software(Altschul et al., 1997) to align various databases such as NR, NT, TrEMBL, SwissProt, COG, GO and KEGG (Ashburner et al., 2000; Yangyang et al., 2006; Tatusov et al., 2000; Kanehisa et al., 2004) with the value of E less than 1e-5. The best hit for each unigene with the highest sequence similarity from the above databases was chosen and the annotations were retrieved. KOG was used to analyze, predict and classify unigenes with probable functions, whereas the Aracyc database was used to assign pathways (Tatusov et al., 2001). The Blast2GO program was used to obtain GO annotation according to molecular function, biological process and cellular component ontologies (Ana and Stefan, 2008; Fan et al., 2006).

4.7. Nucleotide content analysis and identification of SSR markers

The percentage compositions of the nucleotides A, T, G and C were calculated for each sequence and across the entire distribution of transcripts. Simple sequence repeats(SSRs) were detected using the MIcroSAtellite tool. SSRs were detected by considering the 100 bp flanking sequences upstream and downstream of the SSRs. The parameters used for the development of SSRs areprovidedin Table 7.

4.8. Quantitative analysis and functional enrichment analysis of different gene expression

The RPKM value was used to calculate the unigene expression in our study (Grabherr et al., 2011). The unigenes of different gene expression (DGE) in two types of tartary buckwheats were selected by using the Ebseq software with FDR less than 0.01 and Log2 ratio greater than 1 (two-fold change)(Leng et al., 2013) and aligned to GO databases and COG databases with the value of E less than 1e-5.

4.9. Quantitative real-time PCR(qRT-PCR) analysis

Five unigenes involved in flavonoid synthesis were chosen for validation by qRT-PCR. Total RNA was isolated from YTB and BTB using the RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA). For qRT-PCR, 1 μg of total RNA was reverse-transcribed using the Superscript II First Strand Synthesis Kit (Invitrogen, Carlsbad, CA, USA) and an oligo(dT)20 primer. Transcription levels were analyzed by real-time PCR. The gene-specific primer sets were designed for real-time PCR, and the primers are listed in S11 Table. Gene-of-interest expression was normalized to that of the histone H3

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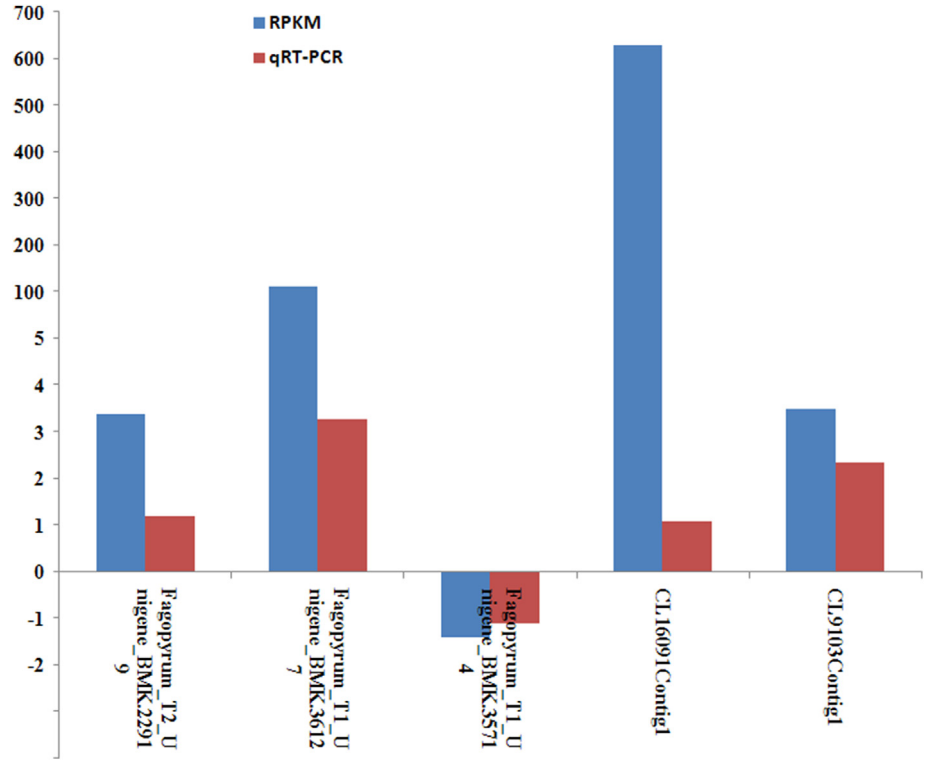

Fig. 14. qRT-PCR validation of RPKM analysis of the five unigenes involved in flavonoid biosynthesis of YTB and BTB.

Table 7
Parameters used for development of SSRs.

S.no	SSR type	Unit size	Minimum number of repeats
1	Mono nucleotide	Repeats 1	10
2	Di nucleotide	Repeats 2	6
3	Tri nucleotide	Repeats 3	5
4	Tetra nucleotide	Repeats 4	5
5	Penta nucleotide	Repeats 5	5
6	Hexa nucleotide	Repeats 6	5

Maximal number of bases interrupting two SSRs in a compound microsatellite:100.

housekeeping gene. Real-time PCR reactions were performed in triplicate on a MiniOption system (Bio-Rad Laboratories, Hercules, CA) with the QuantiTect SYBR Green PCR Kit (Qiagen). The PCR protocol was as follows: denaturation for 5 min at 95 °C, followed by 39 cycles of denaturation for 15 s at 95 °C, annealing for 15 s at 60 °C, and elongation for 20 s at 72 °C. PCR results were calculated as the mean of 3 replicated treatments. Significant differences between treatments were evaluated by standard deviation.

Data availability

The full data sets have been submitted to NCBI, Genebank under Bioproject, Accession: PRJNA271797.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.pbiomolbio.2016.11.003.

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