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ORIGINAL ARTICLE

Genome-wide characterization and analysis of the anthocyanin biosynthetic genes in *Brassica oleracea*

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Abstract

Main conclusion **From *Brassica oleracea* genome, 88 anthocyanin biosynthetic genes were identified. They expanded via whole-genome or tandem duplication and showed significant expression differentiation. Functional characterization revealed *BoMYB113.1* as positive and *BoMYBL2.1* as negative regulators responsible for anthocyanin accumulation.**

Abstract *Brassica oleracea* produces various health-promoting phytochemicals, including glucosinolates, carotenoids, and vitamins. Despite the anthocyanin biosynthetic pathways in the model plant *Arabidopsis thaliana* being well characterized, little is known about the genetic basis of anthocyanin biosynthesis in *B. oleracea*. In this study, we identified 88 *B. oleracea* anthocyanin biosynthetic genes (BoABGs) representing homologs of 46 *Arabidopsis* anthocyanin biosynthetic genes (AtABGs). Most anthocyanin biosynthetic genes, having expanded via whole-genome duplication and tandem duplication, retained more than one copy in *B. oleracea*. Expression analysis revealed diverse expression patterns of BoABGs in different tissues, and BoABG duplications showed significant expression differentiation. Additional expression analysis and functional characterization revealed that the positive regulator *BoMYB113.1* and negative regulator *BoMYBL2.1* may be key genes responsible for anthocyanin accumulation in red cabbage and ornamental kale by upregulating the expression of structural genes. This study paves the way for a better understanding of anthocyanin biosynthetic genes in *B. oleracea* and should promote breeding for anthocyanin content.

Keywords Cabbage · Anthocyanin biosynthetic genes · Comparative genomics · Expression pattern · Functional characterization

Abbreviations

ABGs	Anthocyanin biosynthetic genes
ROS	Reactive oxygen species
PAL	Phenylalanine ammonia lyase
C4H	Cinnamate 4-hydroxylase
4CL	4-Coumarate: CoA ligase
CHS	Chalcone synthase
F3H	Flavanone 3-hydroxylase
F3′H	Flavonoid 3′-hydroxylase
FLS	Flavonols by flavonol synthase
DFR	Dihydroflavonol 4-reductase
ANS	Anthocyanidin synthase
GEO	Gene expression omnibus
WGT	Whole-genome triplication

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Introduction

Anthocyanins are a large group of secondary metabolites belonging to the flavonoids that are widely found in higher plants (Springob et al. 2003; Lepiniec et al. 2006). These metabolites provide plants with red to purple pigments that play important roles in growth and development, attracting pollinators and seed carriers, protecting plants against reactive oxygen species (ROS) and UV-B radiation (Solovchenko and Schmitz-Eiberger 2003; Landi et al. 2014), and mediating plant–microbe interactions (Gould 2004). Additionally, the antioxidant properties of anthocyanins promote human health by protecting against diabetes, cardiovascular disease and cancer (Winkel-Shirley 2001; Nhukarume et al. 2010).

Most anthocyanin genes have been well characterized in the model plant *Arabidopsis thaliana*. Anthocyanins are synthesized through the phenylpropanoid pathway, starting with the conversion of phenylalanine to coumarate-CoA by phenylalanine ammonia lyase (PAL), cinnamate 4-hydroxylase (C4H) and 4-coumarate: CoA ligase (4CL) (Zhang et al. 2014) (Fig. 1). The key enzymes chalcone synthase (CHS), chalcone isomerase (CHI), flavanone 3-hydroxylase (F3H) and flavonoid 3′-hydroxylase (F3′H) catalyze malonyl CoA and ρ-coumaroyl CoA to produce dihydroflavonols (Holton and Cornish 1995). These intermediates are oxidized to flavonols by flavonol synthase (FLS) or catalyzed by the dihydroflavonol 4-reductase (DFR) and anthocyanidin synthase (ANS) genes to produce various anthocyanidins (Harborne and Williams 2001; Winkel-Shirley 2002). The anthocyanidins undergo modification via glycosylation, acylation and/or methylation to form stable anthocyanidins. In *A. thaliana*, eight characterized genes are associated with these different modifications. UGT78D2, UGT75C1, UGT79B1 and UGT84A2 are responsible for glycosylation, and A5G6‴MaT, A3G6″p-CouT, A3G6″p-CouT and SCPL10 are responsible for acylation (Shi and Xie 2014). However, no genes responsible for methylation have been characterized in *A. thaliana*. Anthocyanin biosynthesis is largely regulated at the transcriptional level (Fig. 1). R2R3-MYBs, including MYB11, MYB12, and MYB111, independently positively regulate the phenylpropanoid pathway and early anthocyanin biosynthetic genes (Stracke et al. 2010). Late anthocyanin biosynthetic genes are activated by the complex MYB-bHLH-WDR (MBW), which comprises one of the R2R3-MYB proteins (PAP1, PAP2, MYB113, or MYB114), one of the bHLH proteins (GL3, EGL3, or TT8), and a WD repeat protein (Zhao et al. 2013). The R3-MYB proteins CPC and MYBL2 are characterized as negative regulators interacting with MBW

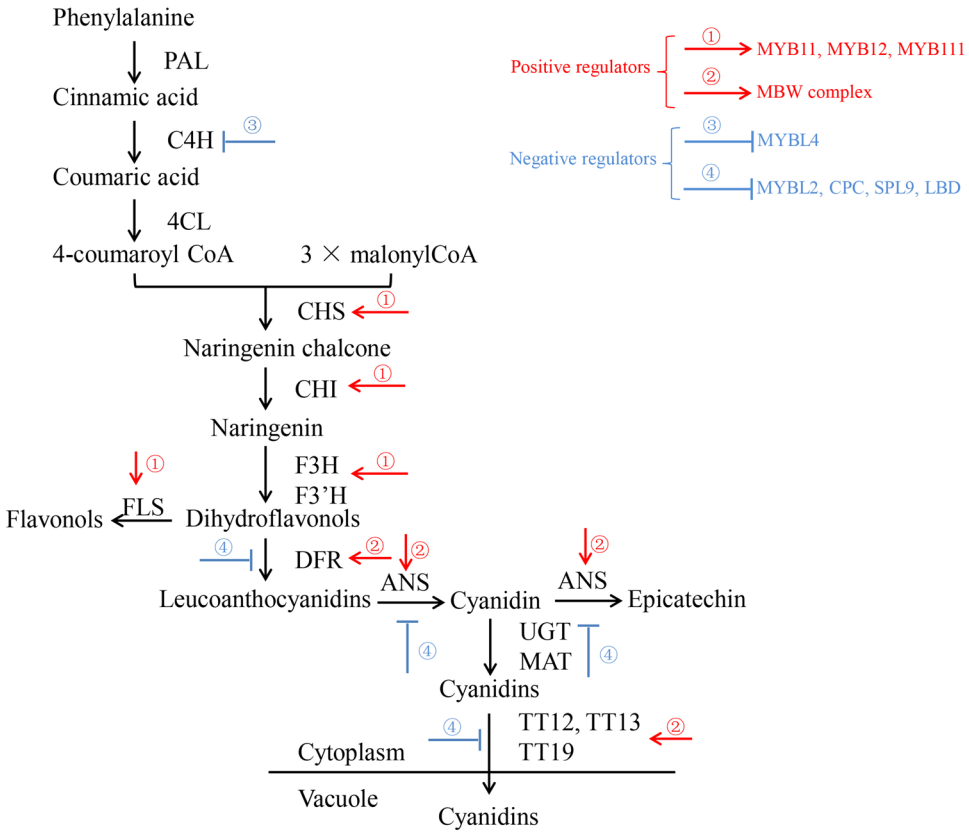

Fig. 1 The biosynthetic pathway of anthocyanin

complexes (Shi and Xie 2014). The other necessary step is the transport of anthocyanins from the cytosol to the vacuole. The important transport genes include TT12, TT19 and AHA10 (TT13) (Zhao and Dixon 2010).

Brassica oleracea comprises multiple important vegetable crops that have contributed to human health and nutrition for hundreds of years (Parkin et al. 2014). The species exhibits diversity in anthocyanin accumulation. Most varieties accumulate anthocyanins at low levels, whereas several cultivars of cauliflower, red cabbage, kale and kohlrabi, are extremely enriched in anthocyanins (Scalzo et al. 2008). However, the mechanisms underlying anthocyanin accumulation in *B. oleracea* are poorly understood. To date, only one gene has been characterized: a R2R3-MYB transcription factor, MYB2 (PAP2-like), responsible for the purple cauliflower mutant (Chiu et al. 2010). Other studies revealed that MYB2 is possible key regulator in red cabbage, kale and kohlrabi, and MYBL2 is negative key regulator in red cabbage, but this hypothesis has not been verified (Song et al. 2018; Yan et al. 2019).

The available *B. oleracea* reference genomes make it possible to perform genome-wide searches for anthocyanin biosynthetic genes based on their orthologs in *A. thaliana*. In the present study, we provide comprehensive information on anthocyanin biosynthetic genes in *B. oleracea* by performing comparative genomic analysis between *B. oleracea* and *A. thaliana*. We also profile the expression patterns in tissues of green and purple plants and further explored the function of positive regulator *BoMYB113.1* and negative regulator *BoMYBL2.1*. This study will improve our understanding of the anthocyanin biosynthetic genes in *B. oleracea*.

Materials and methods

Identification and analysis of ABGs in the *B. oleracea* genome

All the sequences of anthocyanin biosynthetic genes (ABGs) of *A. thaliana* referred to in this study were downloaded from the Col-0 *Arabidopsis* reference genome in TAIR (http://www.arabidopsis.org/). The 02-12 cabbage reference genome sequence (version 1.0) (Liu et al. 2014) and annotated gene sequences from Bolbase (http://www.ocri-genomics.org/bolbase/index.html) were used to identify the ABGs in *B. oleracea.*

To survey ABGs in *B. oleracea*, we performed a comparative genomic analysis between *B. oleracea* and *A. thaliana* using the genome sequences and corresponding annotation information of the two species. The anthocyanin biosynthetic gene and protein sequences of *A. thaliana* were aligned with the genome and protein sequences of *B. oleracea* using BLASTN and BLASTP with an *E*-value cutoff ≤ 1E−10 and coverage ≥ 0.75. The syntenic orthologs between *A. thaliana* and *B. oleracea* from the BRAD were determined based on the following criteria: a sequence similarity of $E \leq 10^{-20}$, the collinearity of flanking genes, and non-syntenic orthologs with a sequence identity > 70% and coverage > 60% (Cheng et al. 2012a, b). In addition, gene clusters were determined according to the description by Alamery et al. (2018). Furthermore, the physical distance between neighboring ABGs was required to be ≤ 200 kb.

Availability of all *B. oleracea* genes in this work

The gene IDs, names, chromosome locations, coding sequences and protein sequences of all *B. oleracea* anthocyanin pathway genes were shown in Supplementary Table S1.

Chromosome distribution analysis

The physical position information of ABGs was downloaded from the *B. oleracea* database. The distribution of genes on the chromosomes was subsequently determined using MapChart software (Voorrips 2002). BoABGs on unanchored scaffolds were displayed independently.

Expression analysis of ABGs in *B. oleracea*

The expression patterns of ABGs in *B. oleracea* were measured using RNA-Seq data. RNA-seq data for seven tissues, namely root, callus, bud, silique, flower, stem and leaf tissues, of *B. oleracea* accession 02-12 were obtained from the Gene Expression Omnibus (GEO) database with the accession number GSE42891.

Total RNA was extracted from leaves of 8-week-old plants of red cabbage line 15Z_P, white cabbage line 120QY_G, and ornamental kale line S2309, and was used for cDNA library construction and RNA sequencing following the protocol as previously described (Han et al. 2018). FPKM values are available in Supplementary Table S2. With *B. oleracea* actin gene (AF044573.1) an internal control, quantitative RT-PCR (qRT-PCR) was performed to validate expression level of BoABGs in 15Z_P and 120QY_G. The qRT-PCR mixture was prepared using SYBR Premix Ex Taq II (Tli RNase H Plus; Takara, Dalian, China), and reactions were conducted on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). All samples were assayed in triplicate. The relative expression levels were calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001). The primers used in this study were listed in Supplementary Table S3.

Agrobacterium tumefaciens-mediated *Arabidopsis* transformation

The coding sequences of *BoMYB113.1* (amplified from red cabbage 15Z_P), and *BoMYBL2.1* (amplified from white cabbage line 120QY_G) and the fragments were sub-cloned into a modified binary vector pBWA(V)BS (reconstructed from pCAMBIA1301) (Han et al. 2019). The constructs were introduced to *A. thaliana* Col-0 plants using the floral dip method (Clough and Bent 1998). Overexpressing transgenic lines were obtained by screening the T0 seeds on MS (Murashige and Skoog) medium containing 30 mg/L of hygromycin and were further confirmed by RT-PCR analysis. Measurement of anthocyanin in the transgenic plants was conducted following the methods previously reported (Wang et al. 2016).

Virus-induced gene silencing (VIGS) of *BoMYB113.1* and *BoMYBL2.1* in cabbage

VIGS using cabbage leaf curl virus (CaLCuV)-based vector was conducted following the methods described by Xiao et al. (2020). In brief, conserved sequences of *BoMYB113.1* (34–533 nt in the coding sequence) and *BoMYBL2.1* (51–550 nt in the coding sequence) were selected, amplified, and inserted to PCVA vector. Empty PCVA, PCVA-BoMYB113.1, PCVA-BoMYBMYBL2.1 and PCVB were independently transformed into *Agrobacterium* GV3101 cells. The *Agrobacterium* culture suspensions of PCVB and PCVA-*BoMYB113.1* were mixed together at a 1:1 ratio for inoculation of red cabbage 15Z_P, PCVB and PCVA-*BoMYBL2.1* were mixed together at a 1:1 ratio for inoculation of white cabbage 120QY_G, and as controls, PCVB and PCVA were mixed together at a 1:1 ratio for inoculation of 15Z_P and 120QY_G. The phenotypes were evaluated 2–3 weeks after sowing in compost.

Statistical analysis

Statistical analysis was performed using Student's *t* test. Significant differences refer to statistical significance at two levels $P < 0.05$ and $P < 0.01$. All the measurements were performed by three independent biological replicates.

Results

Identification of anthocyanin biosynthetic genes in *B. oleracea*

In *A. thaliana*, 51 *Arabidopsis* anthocyanin biosynthetic genes (AtABGs) have been reported, including 29 structural genes encoding anthocyanin biosynthetic enzymes, 19 regulatory genes encoding transcriptional factors, and 3 transport genes that are required for anthocyanin transport from the cytosol to the vacuole (Table 1; Supplementary Table S1). Using the AtABGs as queries, we searched their syntenic and non-syntenic orthologs in *B. oleracea*. A total of 88 *B. oleracea* anthocyanin biosynthetic genes (BoABGs) were identified, representing homologs of 46 of the 51 AtABGs (Table 1; Fig. 2; Supplementary Table S1). The other 5 AtABGs, named *FLS6*, *A3G6"p-CouT1*, *A3G6"p-CouT2*, *SCPL10* and *MYB11*, had no *B. oleracea* orthologs. The BoABG members were named according to the names given in the *A. thaliana* anthocyanin biosynthetic pathway. However, some tandemly duplicated AtABGs showed quite similar sequences, and their *B. oleracea* orthologs could not be distinguished, so they were named according to one of the tandemly duplicated AtABGs. For instance, *B. oleracea* orthologs of *FSL2*, *FSL3*, *FSL4*, and *FSL5* were named *BoFSL2.1*, *BoFSL2.2*, *BoFSL2.3*, *BoFSL2.4* and *BoFSL2.5*, respectively; *MYB113*, *MYB114* and *PAP2* were named *BoMYB113.1*, *BoMYB113.2* and *BoMYB113.3*, respectively. These BoABGs comprise 52 structural genes, 32 transcriptional factors and 4 transport genes. Based on the orthology analysis, 79 (89.0%) BoABGs were syntenic orthologs of 46 AtABGs, and 9 (10.0%) BoABGs had no syntenic relationships.

Genomic distributions of BoABGs

Genome chromosomal location analysis revealed that the BoABGs were distributed on all 9 chromosomes and all three sub-genomes (Fig. 3). Of the 88 BoABGs, 61 were unevenly distributed on the 9 chromosomes, with 4, 6, 9, 8, 6, 8, 5, 8 and 7 ABGs mapped on chromosomes C01-09 in the *B. oleracea* genome, respectively. The remaining 27 genes, representing 30.68% of the total BoABGs, were positioned on different scaffolds, which were not anchored to any chromosome.

Genome rearrangement after WGT in *B. oleracea* led to the following three sub-genomes, with gene densities from high to low: LF, MF1, and MF2 (Liu et al. 2014). With this sub-genomic information, we then assigned BoABGs to the three sub-genomes (Table 1). Of the 79 syntenic orthologs, 33, 19 and 27 were located in LF, MF1 and MF2, respectively.

Duplication of BoABGs

The BoABGs in the genome of *B. oleracea* have expanded mainly due to whole-genome triplication (WGT) since the divergence of the species from *A. thaliana* (Liu et al. 2014). Most of the ABGs syntenic to AtABGs were present in multiple copies in *B. oleracea*. There were thirteen ABGs with ≥ 3 copies in *B. oleracea*, whereas the remaining 33

Table 1 Information of the *Brassica oleracea* anthocyanin biosynthetic genes (BoABGs)

A. thaliana	*B. oleracea*			
	Synteny ortholog			Non-synteny ortholog
	LF	MF1	MF2	
Structural genes				
Biosynthetic genes in phenylpropanoid pathway				
AtPAL1 (AT2G37040)	***BoPAL1.1*** (Bol025522)	***BoPAL1.2*** (Bol037689)	–	***BoPAL1.3*** (Bol005084)
AtPAL2 (AT3G53260)	***BoPAL2.1*** (Bol025102)	***BoPAL2.2*** (Bol041738)	***BoPAL2.3*** (Bol005411)	–
AtPAL3 (AT5G04230)	–	–	***BoPAL3.1*** (Bol005493)	***BoPAL3.2*** (Bol006745)
AtPAL4 (AT3G10340)	***BoPAL4*** (Bol011375)	–	–	–
AtC4H (AT2G30490)	***BoC4H.1*** (Bol006704)	***BoC4H.2*** (Bol033347)	***BoC4H.4*** (Bol004608)	–
		BoC4H.3 (Bol033349)	***BoC4H.5*** (Bol004610)	–
At4CL1 (AT1G51680)	–	–	***Bo4CL1*** (Bol031583)	–
At4CL2 (AT3G21240)	***Bo4CL2.1*** (Bol038385)	–	–	–
	Bo4CL2.2 (Bol038386)			
At4CL3 (AT1G65060)	***Bo4CL3*** (Bol012584)	–	–	–
At4CL5 (AT3G21230)	***Bo4CL5.1*** (Bol038387)	–	***Bo4CL5.3*** (Bol026622)	–
	Bo4CL5.2 (Bol038389)		***Bo4CL5.4*** (Bol026623)	
Early biosynthetic genes				
AtCHS(TT4) (AT5G13930)	***BoCHS.1*** (Bol043396)	***BoCHS.2*** (Bol034259)	***BoCHS.3*** (Bol004244)	–
AtCHI(TT5) (AT3G55120)	***BoCHI.1*** (Bol044343)	–	***BoCHI.3*** (Bol008652)	***BoCHI.4*** (Bol018696)
	BoCHI.2 (Bol044344)	–	–	–
AtF3H (AT3G51240)	***BoF3H.1*** (Bol010585)	–	***BoF3H.2*** (Bol002277)	***BoF3H.3*** (Bol041656)
				BoF3H.4 (Bol044664)
AtF3'H (AT5G07990)	***BoF3'H*** (Bol043829)	–	–	–
AtFLS1 (AT5G08640)	***BoFLS1*** (Bol043773)	–	–	–
AtFSL2 (AT5G63580)	***BoFSL2.1*** (Bol019127)	***BoFlS2.3*** (Bol020738)	***BoFlS2.5*** (Bol019125)	–
	BoFSL2.2 (Bol019128)	***BoFlS2.4*** (Bol020739)		
AtFLS3 (AT5G63590)	–	–	–	–
AtFLS4 (AT5G63595)	–	–	–	–
AtFLS5 (AT5G63600)	–	–	–	–
AtFLS6 (AT5G43935)	–	–	–	–
Late biosynthetic genes				
AtDFR(TT3) (AT5G42800)	–	–	***BoDFR*** (Bol035269)	–
At ANS (AT4G22880)	***BoANS.1*** (Bol014986)	***BoANS.2*** (Bol042059)	–	–
AtUGT79B1 (AT5G54060)	***BoUGT79B1.1*** (Bol038805)	–	–	***BoUGT79B1.2*** (Bol014515)
				BoUGT79B1.3 (Bol014517)
				BoUGT79B1.4 (Bol014519)
AtUGT75C1 (AT4G14090)	–	–	***BoUGT75C1*** (Bol027055)	–
AtUGT78D2 (AT5G17050)	–	–	***BoUGT78D2*** (Bol021317)	–
AtUGT84A2 (AT3G21560)	***BoUGT84A2.1*** (Bol038351)	***BoUGT84A2.2*** (Bol023208)	–	–
At5MAT (AT3G29590)	–	–	***Bo5MAT.1*** (Bol032283)	–
AtA3G6''p-CouT1 (AT1G03940)	–	–	–	–
AtA3G6''p-CouT2 (AT1G03495)	–	–	–	–
AtSCPL10 (AT2G23000)	–	–	–	–

Table 1 (continued)

A. thaliana	*B. oleracea*			
	Synteny ortholog			Non-synteny ortholog
	LF	MF1	MF2	
Regulatory genes				
Positive regulators				
AtMYB11 (AT3G62610)	–	–	–	–
AtMYB12 (AT2G47460)	***BoMYB12.1*** (Bol001533)	***BoMYB12.2*** (Bol002581)	***BoMYB12.3*** (Bol029626)	–
AtMYB111 (AT5G49330)	***BoMYB111.1*** (Bol016599)	***BoMYB111.2*** (Bol033054)	***BoMYB111.3*** (Bol032351)	–
AtPAP1 (AT1G56650)	–	–	***BoPAP1*** (Bol045347)	–
AtPAP2 (AT1G66390)	–	–	–	–
AtMYB113 (AT1G66370)	***BoMYB113.1*** (Bol012528) ***BoMYB113.2*** (Bol012531)	–	***BoMYB113.3*** (Bol042409)	–
AtMYB114 (AT1G66380)	–	–	–	–
AtTT2(AT5G35550)	–	–	***BoTT2*** (Bol014029)	–
AtTT8 (AT4G09820)	***BoTT8*** (Bol004077)	–	–	–
AtGL3 (AT5G41315)	***BoGL3*** (Bol014556)	–	–	–
AtEGL3 (AT1G63650)	–	***BoEGL3.1*** (Bol022614)	***BoEGL3.2*** (Bol029662)	–
AtTTG1 (AT5G24520)	***BoTTG1*** (Bol022420)	–	–	–
Negative regulators				
AtMYBL2 (AT1G71030)	***BoMYBL2.1*** (Bol016164)	***BoMYBL2.2*** (Bol034966)	–	–
AtMYB4 (AT4G38620)	–	***BoMYB4.1*** (Bol018511)	***BoMYB4.2*** (Bol016066)	–
AtCPC (AT2G46410)	***BoCPC.1*** (Bol000928)	***BoCPC.2*** (Bol021780)	***BoCPC.3*** (Bol029590)	–
AtSPL9 (AT2G42200)	***BoSPL9.1*** (Bol004847)	***BoSPL9.2*** (Bol002678)	–	–
AtLBD37 (AT5G67420)	***BoLBD37.1*** (Bol014304)	***BoLBD37.2*** (Bol008082)	***BoLBD37.3*** (Bol005707)	–
AtLBD38 (AT3G49940)	***BoLBD38.1*** (Bol007980)	***BoLBD38.2*** (Bol021982)	***BoLBD38.3*** (Bol016975)	–
AtLBD39 (AT4G37540)	–	–	–	***BoLBD39*** (Bol019060)
Transport genes				
AtTT12 (AT3G59030)	–	–	***BoTT12*** (Bol023767)	–
AtTT13 (AT1G17260)	–	**BoTT13** (Bol029307)	–	–
AtTT19 (AT5G17220)	***BoTT19.1*** (Bol019821)	–	***BoTT19.2*** (Bol021325)	–

ABGs had 1–2 syntenic orthologs due to gene fractionation after the triplication event (Table 1).

In addition to the WGT-derived BoABGs, some tandem duplications were identified. According to the gene cluster defined by Alamery et al. (2018), we identified 21 tandemly duplicated BoABGs located in 10 gene clusters, with 2 clusters each on C03 and C05, one each on C04, C06 and C08, and three on scaffolds (Scaffold000133, Scaffold000121_P2 and Scaffold000173), whereas the remaining 67 genes were found as singletons in chromosomes (Fig. 3). The numbers of genes in clusters ranged from two to three in *B. oleracea*. *A. thaliana* and *B. oleracea* shared some common tandemly duplicated loci, e.g., *MYB113*, *MYB114* and *PAP1*, indicating that these tandem duplications occurred before the divergence of the two species. We also identified tandem duplications solely present in *B. oleracea*; for instance, *C4H*, *4CL5*, *CHI*, *F3H* and *AtUGT79B1* produced tandemly duplicated copies in different sub-genomes (note: *Bo4CL2.1* and *Bo4CL2.2* possibly belong to one gene, in which case they were incorrectly predicted as belonging to two genes in the 02-12 reference genome, so *Bo4CL2.1* and *Bo4CL2.2* were not considered tandem duplications). All the tandem duplications were biosynthetic structural genes.

Expression profiles of BoABGs in various tissues

Most *B. oleracea* varieties accumulate low levels of anthocyanins, so we chose a white cabbage variety '02-12' with a low level of anthocyanins to analyze the expression profiles of BoABGs.

Based on RNA-seq data of cabbage variety '02-12' from the GEO database (GSE42891), the expression levels of all 88 BoABGs in different tissues, including root, callus, bud, silique, flower, stem and leaf tissues, were analyzed. A hierarchical map was constructed to show the abundance of the BoABGs in the seven tissues (Fig. 4, Supplementary Table S2). According to the RNA-seq data, the expression of

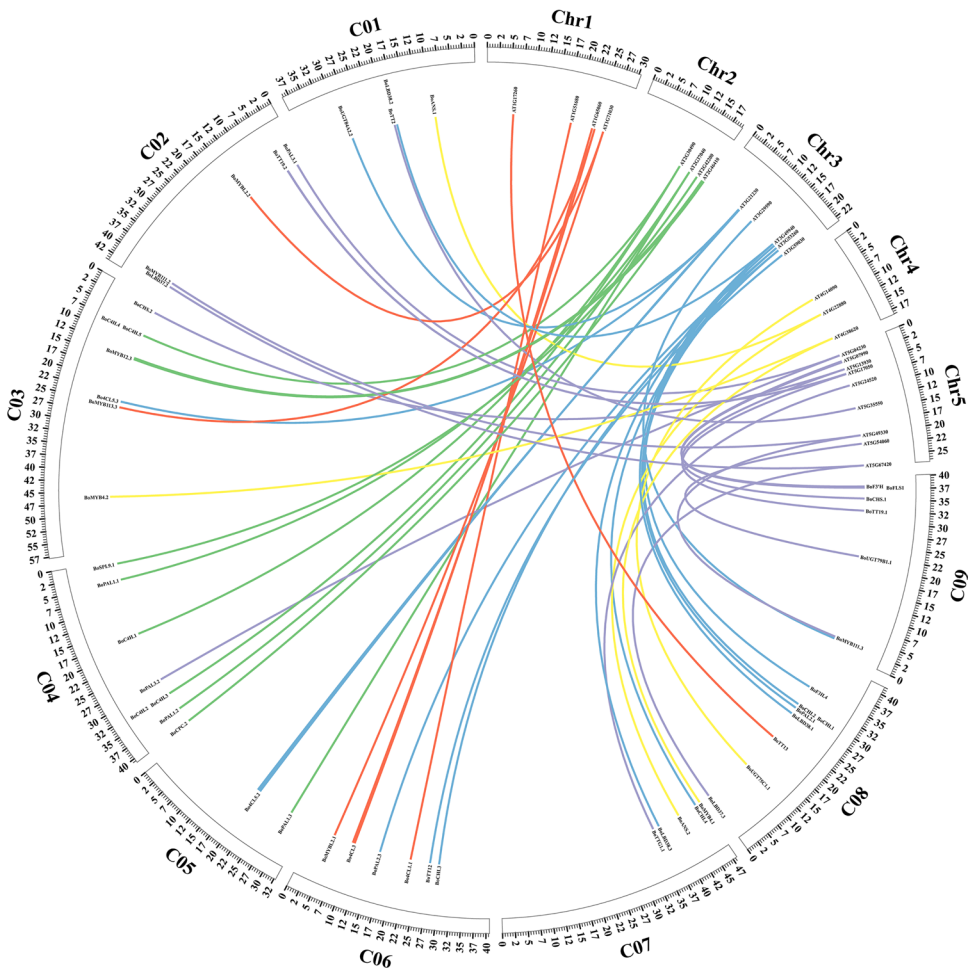

Fig. 2 Circos diagram of syntenic anthocyanin biosynthetic genes in *Arabidopsis thaliana* and *Brassica oleracea*. Chr1 to Chr5 indicate *A. thaliana* chromosomes, and C01 to C09 indicate *B. oleracea* chromosomes

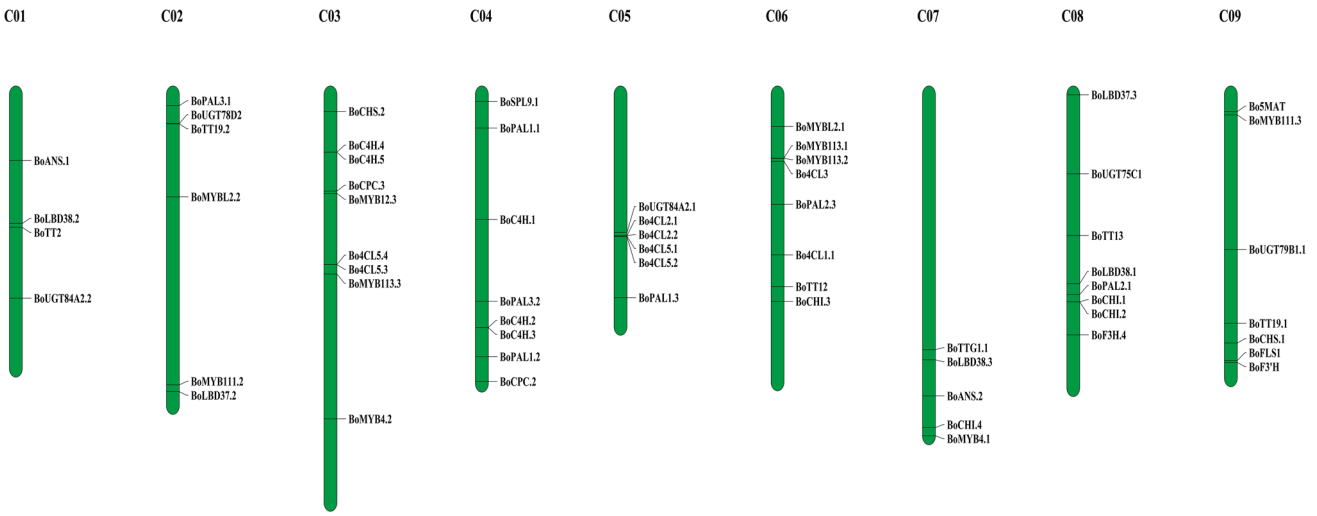

Fig. 3 Genomic localization of the BoABGs on the 9 chromosomes of *B. oleracea*. C01 to C09 indicate *B. oleracea* chromosomes

BoABG members was highly variable among tissues, supporting the diversification of functions for the ABGs during *B. oleracea* development. There were 11 genes showing no expression in any of these seven tissues, which suggested that the genes are nonfunctional or inactive in this variety. Forty BoABGs were expressed in all seven tissues

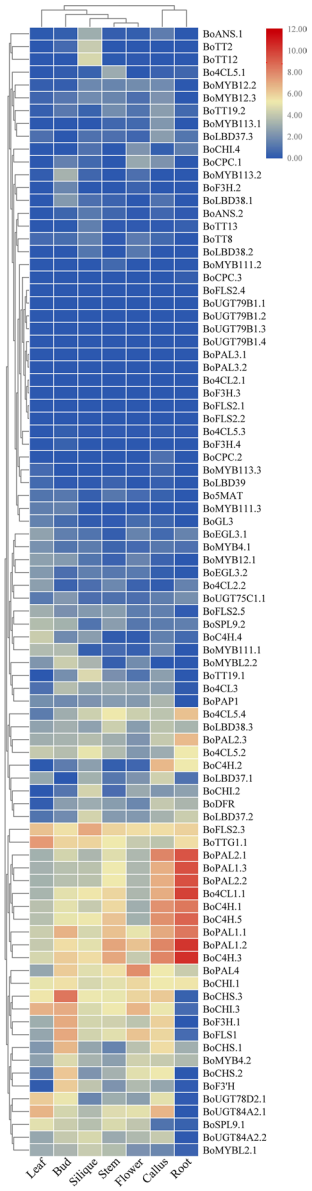

Fig. 4 Expression profiles of BoABGs in seven organs of cabbage '02-12'. The expression level was indicated by a color scale representing FPKM-normalized log2-transformed counts. Red indicates high expression level, and blue indicates low level

(FPKM > 0), and 18 genes showed constitutive expression (FPKM > 2 in all tissues), suggesting that BoABGs play roles at multiple developmental stages. Some genes, such as *BoF3H.4*, *BoMYB111.2*, *BoCPC.2* and *BoCPC.3*, had very low expression levels (FPKM < 1), suggesting that they are less important for growth and development in *B. oleracea.* The root had the highest overall expression level, and the silique had the lowest overall expression level, although it was only slightly lower than that in the leaf. Interestingly, the root had the maximum number of unexpressed genes (39), with 20 lowly expressed genes (FPKM < 1), which included most of the early biosynthetic genes, late biosynthetic genes, transport genes and positive regulatory genes. However, some genes in the root, such as *BoPAL2.1*, *BoPAL1.3*, *BoPAL2.2*, *Bo4CL1.1*, *BoC4H.1*, *BoC4H.5*, *BoPAL1.1*, *BoPAL1.2*, and *BoC4H.3*, were much more highly expressed in the root and callus than in the other tissues. Above all, the expression in tissues indicated that members of the BoABGs might participate in different biological processes in *B. oleracea*.

In different processes of the biosynthetic pathway, most phenylpropanoid pathway genes and early biosynthetic genes showed high expression levels, which may indicate their activity in other branches of the flavonoid pathway. Most early biosynthetic genes, late biosynthetic genes, transport genes and positive regulatory genes showed low expression levels, whereas some negative regulatory genes (*BoMYBL2.1*, *BoSPL9.1*, *BoMYB4.2* and *BoLBD37.2*) had high expression levels.

We also analyzed the expression variance among duplicated ABGs in *B. oleracea* (Fig. 5 Supplementary Table S2). The BoABGs that had more than one copy were selected to analyze the expression variance in *B. oleracea.* Analysis of RNA-Seq data generated from different *B. oleracea* tissues suggested that BoABG duplications had significant expression differentiation. In some duplicated BoABG pairs, such as those of *BoPAL3*, *Bo4CL2*, *BoCHS*, *BoUGT79B1*, *BoMYB12*, *BoMYB113*, *BoEGL3*, *BoMYBL2*, *BoCPC3*, *BoSPL9* and *BoLBD38*, the differential expression levels between the copies were not significant, and most of them had low expression levels. For other genes, such as *BoPAL1*, *BoPAL2*, *BoC4H*, *Bo4CL5*, *BoCHI*, *BoF3H*, *BoFLS*, *BoANS*, *BoUGT84A2*, *BoMYB111*, *BoMYB4*, *BoLBD37* and *BoTT19*, the expression levels differed significantly between the copies. For the duplicated ABGs residing in the same cluster, such as *BoC4H.2* and *BoC4H.3*, *BoC4H.4* and *BoC4H.5*, *Bo4CL5.1* and *Bo4CL5.2*, *Bo4CL5.3* and B*o4CL5.4*, *BoCHI.1* and *BoCHI.2*, and *BoFLS2.3* and *BoFLS2.4*, one copy was much more highly expressed than the other.

Expression analysis of BoABGs in cultivars of red cabbage and ornamental kale with high levels of anthocyanins

Some *B. oleracea* varieties accumulate high levels of anthocyanins, such as red cabbage and some cultivars of ornamental kale. In contrast to the accumulation of anthocyanins in purple cauliflower, which is controlled by a semidominant locus (Chiu et al. 2010), that in red cabbage is believed to be controlled by multiple loci. We analyzed transcriptome data from the leaves of the red cabbage line 15Z-P (high anthocyanin accumulation) and compared them with transcriptome data from the white cabbage line 120QY-G (low anthocyanin accumulation)

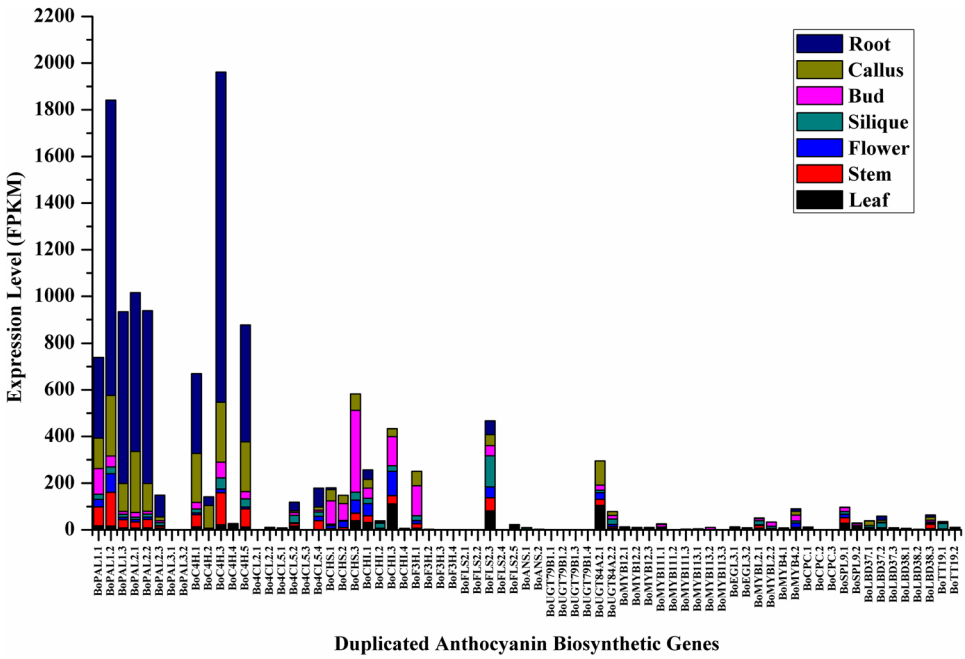

Fig. 5 Expression levels of duplicated anthocyanin biosynthetic genes in *B. oleracea*

(Fig. 6; Supplementary Table S2). Almost all the BoABGs involved in the phenylpropanoid pathway showed lower expression levels in 15Z-P than in 120QY-G. However, the early biosynthetic genes, late biosynthetic genes and transport genes (*BoTT19.1* and *BoTT19.2*) were largely upregulated, consistent with the upregulation of positive regulators, especially *BoMYB113.1* and *BoTT8*, and the downregulation of the negative regulators *BoMYBL2.1* and *BoMYBL2.2*. We selected some belonging to biosynthetic genes, transport genes and regulator genes for qRT-PCR validation, which suggested that expression levels of most genes were in accordance with the transcriptome data (Fig. 7). The results indicated that regulatory genes may be key genes responsible for the high level of anthocyanins in red cabbage.

We selected another plant material, an ornamental kale cultivar with triple-type (green, white, and purple from outer to inner) leaves (Fig. 6). All the BoABGs involved in the phenylpropanoid pathway were upregulated in the purple leaves, in accordance with the upregulation of their positive regulators *BoMYB12.1* and *BoMYB111s*. Among the early biosynthetic genes, *BoC4H.3*, *Bo4CL3*, *BoCHSs*, *BoCHIs*, *BoF3H.1*, *BoF3'H*, *BoFLS1*, and *BoFLS2.3* were upregulated. Among the late biosynthetic genes, *BoDFR*, *BoANSs*, *BoUGT75C1*, and *BoUGT84A2s* and the transport gene *BoTT19s* were upregulated, in accordance with the upregulation of the positive regulators *BoMYB113.1* and *BoTT8* and downregulation of the negative regulators *BoMYBL2.2* and *BoLBD37s*.

These results indicated that the red cabbage and ornamental kale varieties differed in their phenylpropanoid pathways. However, they shared the activation of later biosynthetic processes by the positive regulators *BoMYB113.1* and *BoTT8* and negative regulator *BoMYBL2*.

Functional characterization of *BoMYB113.1* and *BoMYBL2.1*

We introduced *BoMYB113.1* and *BoMYBL2*.1 driven by the CaMV35S promoter into *A. thaliana* Col-0 wild-type plants, respectively. A total of 16 independent *BoMYB113.1-OE* lines and 18 *BoMYBL2.1-OE* lines were generated. In comparison with the brown seeds of wild-type plants, T2 transgenic lines of *BoMYB113.1-OE* had dark brown seeds and *BoMYBL2.1-OE* had light yellow seeds (Fig. 8a). Getting rid of carotenoid and chlorophyll pigments from seedlings by Norflurazon [(4-chloro-5-(methylamino)-2-[3-(trifluoromethyl)phenyl]-3(2H)-pyridazinone] enables a better visualization of anthocyanin. After treatment with Norflurazon, the *BoMYB113.1-OE* seedlings displayed increased level of purple anthocyanin, whereas *BoMYBL2.1-OE* seedlings displayed reduced level of purple anthocyanin (Fig. 8a). *BoMYB113.1-OE* seedlings without Norflurazon treatment also showed obvious over-accumulation of purple anthocyanins on leaves, petioles, veins and hypocotyls. However, less purple phenotype could be distinguished only on hypocotyls of *BoMYBL2.1-OE* seedlings. We

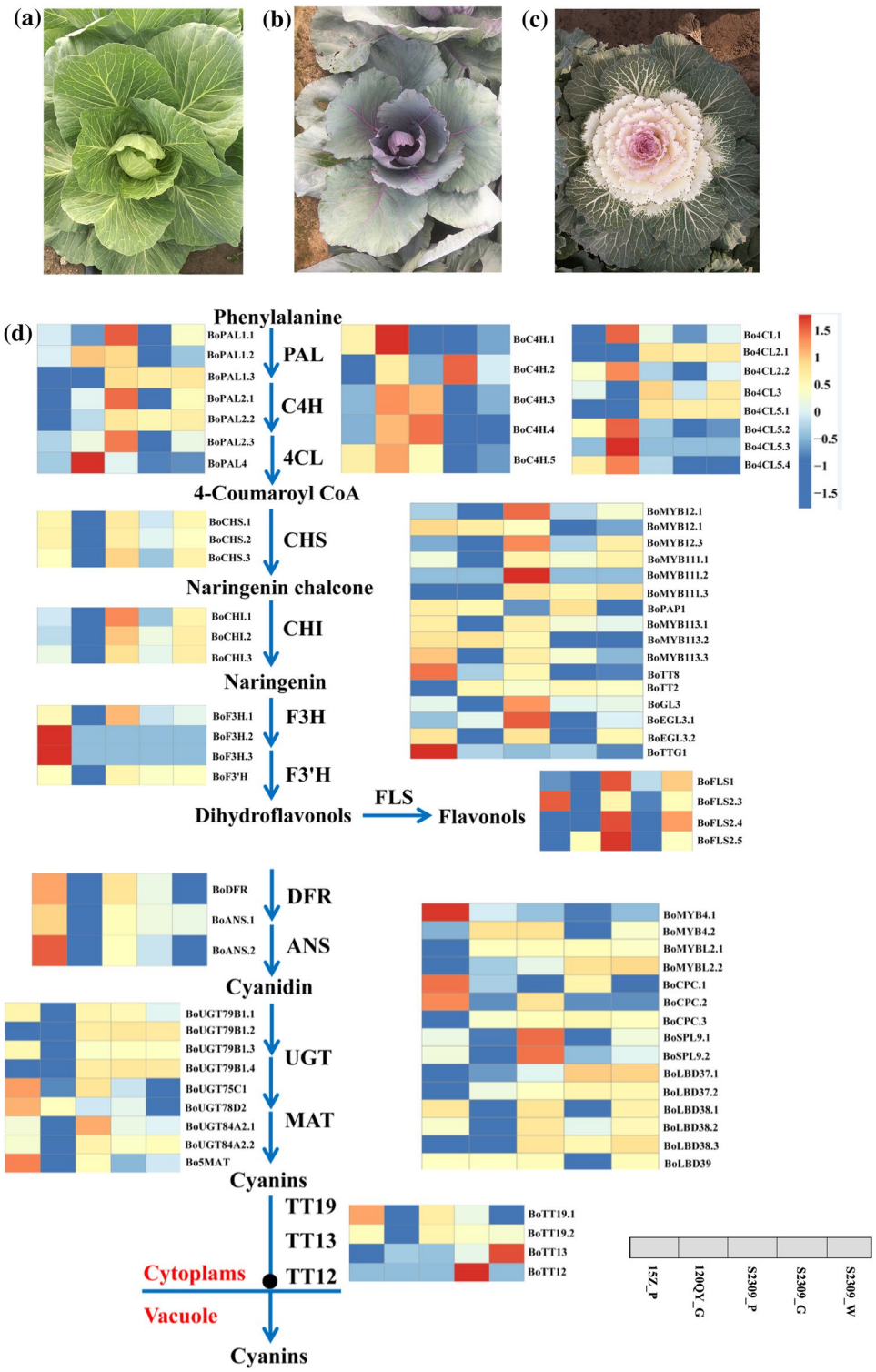

Fig. 6 Expression levels of BoABGs in red cabbage and ornamental kale. **a** Phenotype of the white cabbage cultivar 120QY-G. **b** Phenotype of the red cabbage cultivar 15Z-P. **c** Phenotype of the ornamental kale cultivar S2309. **d** The biosynthetic pathway of anthocyanin and expression levels of BoABGs in leaves of 120QY-G, 15Z-P and S2309. S2309-P indicates the inner purple leaf; S2309-W indicates the middle white leaf, S2309-G indicates the outer green leaf

selected *BoMYB113.1-OE#4* with the highest abundance of *BoMYB113.1* and *BoMYBL2.1-OE#12* with the highest abundance of *BoMYBL2.1* for measurement of anthocyanin contents. Direct quantification of anthocyanin levels confirmed the higher anthocyanin levels in *BoMYB113.1-OE#4* and lower anthocyanin levels in *BoMYBL2.1-OE#12* (Fig. 8b). These results indicated that *BoMYB113.1* was a functional positive regulator and *BoMYBL2.1* is a functional negative regulator of anthocyanin biosynthesis.

As *BoMYB113.1* is a positive regulator of anthocyanin biosynthesis showing high expression level in red cabbage and *BoMYBL2.1* is a negative regulator showing

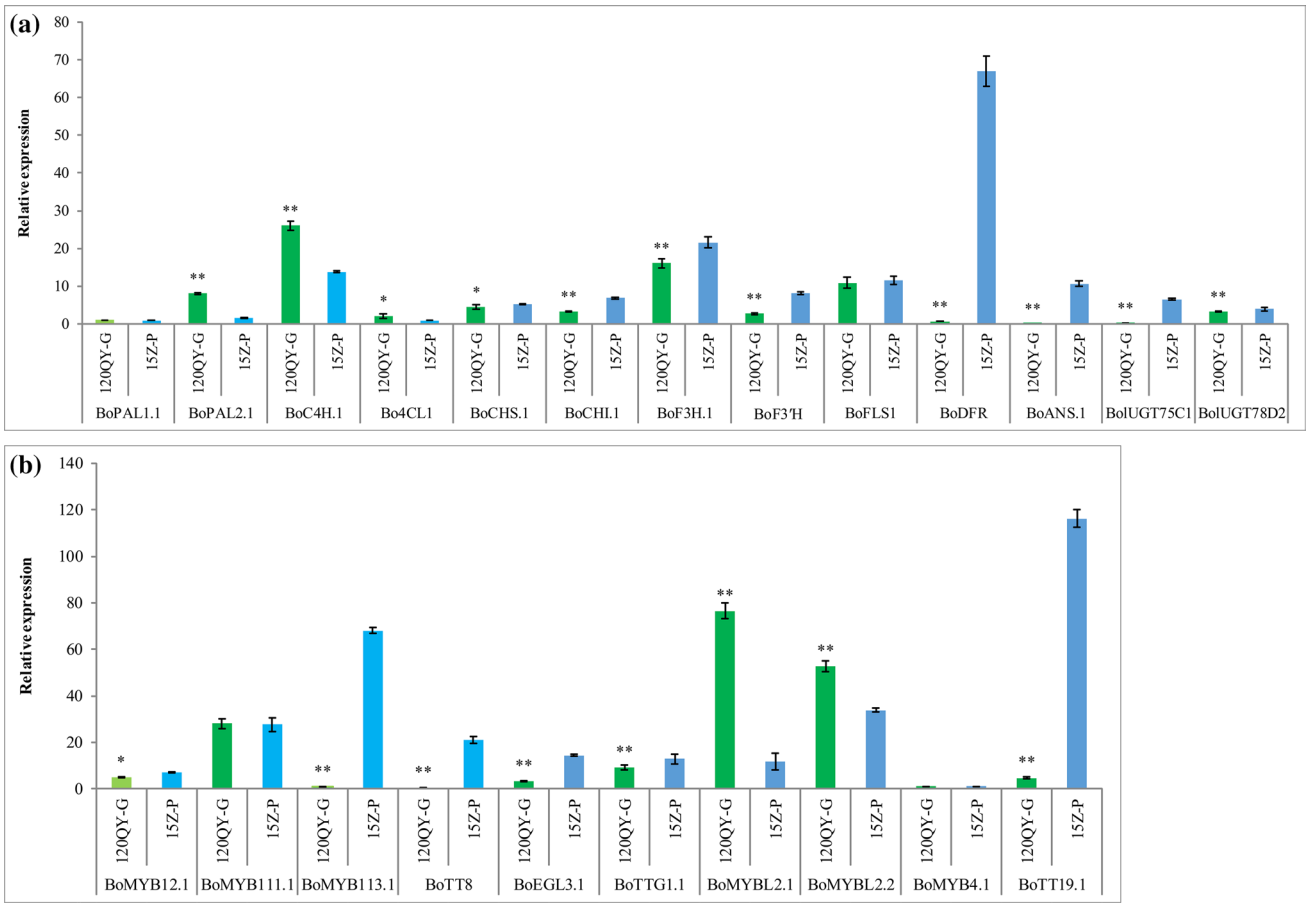

Fig. 7 Quantitative RT-PCR analysis of BoABGs expression in leaves of red cabbage 15Z-P and white cabbage 120QY-G. **a** Expression levels of structural BoABGs genes in 15Z-P and 120QY-G. **b** Expression levels of regulator and transport genes in 15Z-P and 120QY-G. Significant difference at $P_{0.05}$ level is marked by *, and difference at $P_{0.01}$ level is marked by **

high expression level in white cabbage. We conducted VIGS of *BoMYB113.1* in red cabbage 15Z_P, and VIGS of *BoMYBL2.1* in white cabbage 120QY_G using the CaLCuV-based VIGS system described previously (Xiao et al. 2020). The control 15Z_P plants showed obvious purple on hypocotyls, leaf veins and young leaves, whereas some *BoMYB113.1*-silenced plants showed less purple on true leaves and leaf veins (Fig. 8d). To investigate whether the phenotype correlated with reduced expression of *BoMYB113.1* by VIGS, the expression levels of *BoMYB113.1* were measured by qRT-PCR which showed that *BoMYB113.1* was significantly downregulated in *BoMYB113.1*-silenced plants compared with the control plants (Fig. 8c). However, we observed the *BoMYBL2.1*-silenced plants showed no distinguishable differences with the control plants (Fig. 8d). We speculated that *BoMYBL2.1* had minor function in anthocyanin accumulation in red/white cabbage, or it may function together with other genes, in consideration of red phenotype in cabbage is a quantitative character controlled by multiple genes.

Discussion

Brassica oleracea contains various health-promoting phytochemicals, including anthocyanins, glucosinolates, carotenoids, and vitamins. However, little is known about the genetic basis of anthocyanin biosynthesis in *B. oleracea*. In this study, 88 anthocyanin biosynthetic genes in *B. oleracea* were identified by comparative genomic analyses between *A. thaliana* and the cabbage variety 02-12. Most of the AtABGs in *A. thaliana* have orthologous BoABGs in *B. oleracea*, while five AtABGs, namely, *FLS6*, *A3G6"p-CouT1*, *A3G6"p-CouT2*, *SCPL10*, and *MYB11*, had no *B. oleracea* orthologs, indicating that these genes are not necessary for the biosynthesis of anthocyanins.

The number of gene copies may increase through WGD, tandem duplication, segmental duplication, and gene transposition duplication (Sémon and Wolfe 2007). The ABGs in cabbage outnumbered those in *Arabidopsis*. *A. thaliana* and *Brassica* species are derived from a common ancestor. After divergence from *A. thaliana*, *Brassica* genomes experienced

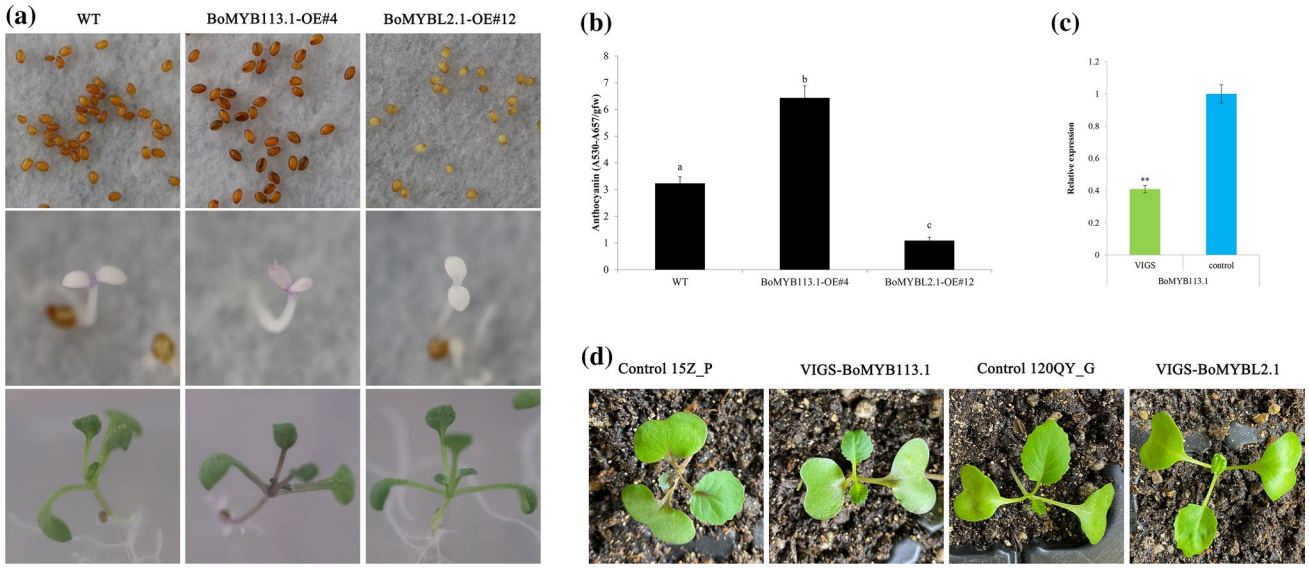

Fig. 8 Functional characterization of *BoMYB113.1* and *BoMYBL2.1* in *A. thaliana* and cabbage. **a** *A. thaliana* seeds and seedlings showing over-accumulation of purple anthocyanins in *BoMYB113.1-OE* lines and down-accumulation of purple anthocyanins in *BoMYBL2.1-OE* lines compared with WT; The non-green seedlings were treated with norflurazon to get rid of carotenoid and chlorophyll pigments; the green seedlings were grown on normal MS plates. **b** Measurement of anthocyanins in *A. thaliana* WT and transgenic seedlings. Values are means ± SD of three biological replicates. The three groups a, b and c show significant differences with each other ($P < 0.01$). **c** Expression of *BoMYB113.1* in control and *BoMYB113.1*-silenced cabbage plants. Significant difference at $P_{0.01}$ level is marked by **. **d** The *BoMYB113.1*-silenced cabbage showed down-accumulation of purple anthocyanins; The *BoMYBL2.1*-silenced cabbage showed no distinguishable differences comparing with the control

a unique whole-genome triplication event (Wang et al. 2011), producing many WGT-derived AtABGs. However, polyploidization after WGD is usually accompanied by gene fractionation and loss (Freeling and Thomas 2006), so most ABGs retained fewer than three WGT-derived copies in the cabbage variety 02-12. Interestingly, the negative regulator of BoABGs retained more WGT-derived copies.

In addition to WGDs, genes may also be duplicated through tandem duplication. Some of these genes remain clustered, which may contribute to the expansion of gene families and facilitate the evolutionary process (Cheng et al. 2012a, b). We identified 10 tandem gene clusters comprising 21 tandemly duplicated BoABGs. Only five tandemly duplicated BoABGs, namely *C4H*, *4CL5*, *CHI*, *F3H* and *AtUGT79B1*, which were biosynthetic structural genes, were produced after the divergence from *Arabidopsis*.

Anthocyanins are derived from branches of the flavonoid pathway, which starts with phenylalanine via the general phenylpropanoid pathway. The biosynthetic genes can be divided into phenylpropanoid pathway genes, early biosynthetic genes, late biosynthetic genes, transport genes and (positive and negative) regulatory genes (Shi and Xie 2014). Transcriptome analyses of the BoABGs using the cabbage variety 02-12 as the material revealed diverse expression patterns of the BoABGs. We found that before the side branch of anthocyanins was activated, the phenylpropanoid pathway genes and early biosynthetic genes had high expression levels. The phenylpropanoid pathway genes *BoPAL2.1*, *BoPAL1.3*, *BoPAL2.2*, *Bo4CL1.1*, *BoC4H.1*, *BoC4H.5*, *BoPAL1.1*, *BoPAL1.2*, and *BoC4H.3* were abundant in all tissues and had high expression levels in the root and callus. The early biosynthetic genes *BoCHS.3*, *BoCHI.3*, *BoF3H.1*, *BoFLS1*, *BoCHS.1*, *BoCHS.2*, and *BoF3'H* had high expression levels in the bud and very low expression levels in the root. However, most of the late biosynthetic genes and positive regulators were expressed at low levels in all tissues, which may be the reason for the low anthocyanin levels in white cabbage. We also found that some duplicated BoABG genes showed significantly different expression patterns, an indicator of non-functionalization or neo-functionalization after gene duplication (Ganko et al. 2007).

Several cultivars of *B. oleracea* are rich in anthocyanins (Scalzo et al. 2008). Chiu et al. (2010) demonstrated that the activation of MYB2 (*BoMYB113.1* in this study) is responsible for the formation of purple cauliflower. Yan et al. (2019) reported that this gene was also responsible for the formation of purple kale, kohlrabi, and cabbage, but this role was not functionally verified. Song et al. (2018) reported that *BoMYBL2-1* (*BoMYBL2.1* in this study) was a negative regulator in purple *B. oleracea* var. *capitata*. We used transcriptome data from red cabbage and a triple-leaf-type (green, white, and purple leaves from outside to inside) ornamental kale as materials to reveal the expression of BoABGs in these accessions. The results revealed

that the positive regulators *BoMYB113.1* and *BoTT8* and the negative regulator *BoMYBL2.1* may be key genes responsible for anthocyanin accumulation by upregulating early biosynthetic genes, late biosynthetic genes and transport genes. The function of *BoMYB113.1* and *BoMYBL2.1* was further confirmed by ectopic expression in *Arabidopsis*. In ornamental kale, *BoMYB12.1* and *BoMYB111s* may also be key regulators by activating the phenylpropanoid pathway genes. Additionally, although *SPL9* was reported as a negative regulator, *BoSPL9s* showed higher expression levels in red tissues than in non-red tissues of red cabbage and ornamental kale, indicating that this gene is not important for anthocyanin accumulation in *B. oleracea*.

Conclusion

Based on whole-genome comparative analysis between *A. thaliana* and *B. oleracea*, 88 anthocyanin biosynthetic genes were identified in *B. oleracea*. Most BoABGs presented multiple copies originating via WGD, tandem duplication and gene loss after the WGT event. We identified 21 tandemly duplicated BoABGs located in 10 gene clusters, most of which formed before the divergence of *A. thaliana* and *B. oleracea*.

Expression analysis revealed diverse expression patterns of BoABGs in different tissues and the expression differentiation of BoABG duplications, indicating non-functionalization or neo-functionalization of the duplications after polyploidization. Expression analysis of BoABGs in red cabbage and ornamental kale revealed that the positive regulators *BoMYB113.1*, *BoTT8*, *BoMYB12.1* and *BoMYB111s* and negative regulator *BoMYBL2* may be key genes responsible for anthocyanin accumulation by upregulating phenylpropanoid pathway genes, early biosynthetic genes, late biosynthetic genes and transport genes. Functional characterization by ectopic expression in *A. thaliana* confirmed the positive role of *BoMYB113.1* and negative role of *BoMYBL2.1* in anthocyanin accumulation; and by VIGS in cabbage further confirmed the positive role of *BoMYB113.1* in anthocyanin accumulation. The results of this study should improve our understanding of the anthocyanin biosynthetic genes in *B. oleracea*.

Author contribution statement HL and ZF conceived and designed the work. FH and XZ performed the experiments and analyzed the data. FH and XZ wrote and revised the manuscript. YZ, YL, ZL, LY, MZ, and YW analyzed the data and revised the manuscript. All authors have read and approved the final manuscript.

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Data availability The data generated or used in this study are included in this article and its supplementary materials. RNA-Seq data were obtained from the Gene Expression Omnibus (GEO) database with the accession number GSE42891. BoABGs sequences were retrieved from Bolbase (http://www.ocri-genomics.org/bolbase/index.html). *Arabidopsis* ABGs sequences were retrieved from Col-0 *Arabidopsis* reference genome in TAIR (http://www.arabidopsis.org/).

Declarations

Conflict of interest The authors declare no competing financial interest.

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