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# Functional characterization of a heterologously expressed *Brassica napus WRKY41-1* transcription factor in regulating anthocyanin biosynthesis in *Arabidopsis thaliana*

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ABSTRACT

Previous studies have shown that a plant WRKY transcription factor, WRKY41, has multiple functions, and regulates seed dormancy, hormone signaling pathways, and both biotic and abiotic stress responses. However, it is not known about the roles of AtWRKY41 from the model plant, *Arabidopsis thaliana*, and its ortholog, BnWRKY41, from the closely related and important oil-producing crop, *Brassica napus*, in the regulation of anthocyanin biosynthesis. Here, we found that the *wrky41* mutation in *A. thaliana* resulted in a significant increase in anthocyanin levels in rosette leaves, indicating that AtWRKY41 acts as repressor of anthocyanin biosynthesis. RNA sequencing and quantitative real-time PCR analysis revealed increased expression of three regulatory genes *AtMYB75*, *AtMYB111*, and *AtMYBD*, and two structural genes, *AT1G68440* and *AtGSTF12*, all of which contribute to anthocyanin biosynthesis, in the sixth rosette leaves of *wrky41-2* plants at 20 days after germination. We cloned the full length complementary DNA of *BnWRKY41-1* from the C2 subgenome of the *B. napus* genotype Westar and observed that, when overexpressed in tobacco leaves as a fusion protein with green fluorescent protein, BnWRKY41-1 is localized to the nucleus. We further showed that overexpression of *BnWRKY41-1* in the *A. thaliana wrky41-2* mutant rescued the higher anthocyanin content phenotype in rosette leaves of the mutant. Moreover, the elevated expression levels in *wrky41-2* rosette leaves of several important regulatory and structural genes regulating anthocyanin biosynthesis were not observed in the *BnWRKY41-1* overexpressing lines. These results reveal that BnWRKY41-1 has a similar role with AtWRKY41 in regulating anthocyanin biosynthesis when overexpressed in *A. thaliana*. This gene represents a promising target for genetically manipulating *B. napus* to increase the amounts of anthocyanins in rosette leaves.

## 1. Introduction

Flavonoids, a class of secondary metabolites found in higher plants, can be categorized into several major groups, and the model plant *Arabidopsis thaliana* contains three types of flavonoids: anthocyanins, flavonols, and proanthocyanidins [1]. Anthocyanins, which exhibit antioxidant properties, not only play important roles in protecting plants against external biotic and abiotic stresses [2–6], but also serve as beneficial micronutrients in the human diet, helping prevent many diseases [7–10]. Elevating anthocyanin levels in crops is therefore considered an important target for plant breeders.

Oilseed rape (*Brassica napus* L., AACC) is grown worldwide, and is an important source of edible oil in the human diet, feed for livestock, and raw materials for industry. There are therefore many socioeconomic reasons for studying its growth, development and associated biological processes, including anthocyanin biosynthesis. Molecular breeding provides an effective means to enhance metabolites levels in crops and there is considerable interest in identifying the genes that govern anthocyanin accumulation, thereby revealing potential targets for crop improvement.

Candidate genes for regulating anthocyanin levels include transcription factors in the WRKY family. The *A. thaliana* genome is predicted to encode a 74 member *W*RKY protein family [11], each of which consists of one or two conserved WRKYGQK motifs at the N-terminus, and a zinc-finger motif at the C-terminus [12]. In *A. thaliana*, WRKY transcription factors can be classified into three different groups, on the basis of both the number of WRKY domains and features of the zinc finger-like motif. Proteins with two WRKY domains belong to

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group I, whereas those with one WRKY domain belong to groups II and III. In general, the zinc finger-like motif of groups I and II has the conserved pattern C-$X_{4-5}$-C-$X_{22-23}$-H-$X_1$-H, while members of group III have a C-X7-C-X23-H-X-C zinc finger-like motif [13]. Previous studies have revealed that WRKY proteins are involved in the regulation of biotic and abiotic stresses [14–16] as well as many developmental and physiological processes, such as root growth [17,18], leaf development [19], trichome formation [20], seed development [20,21], sesquiterpene biosynthesis [22], and senescence [19,23,24]. In addition, there are several reports that WRKY proteins are involved in the control of anthocyanin biosynthesis. For example, GbWRKY1 from *Gossypium barbadense* was demonstrated to inhibit anthocyanin deposition when expressed in *A. thaliana* [25]. The WRKY protein PhPH3 from *Petunia hybrida*, which is closely related to *A. thaliana* AtWRKY44, interacts with WD40-bHLH-MYB complexes when expressed in *A. thaliana*, thereby affecting anthocyanin biosynthesis [26]. It has also been reported that AtWRKY75 represses anthocyanin accumulation in *A. thaliana* seedlings in response to phosphate (Pi) deprivation [17]. Recently, studies indicated that AtWRKY41 not only promotes seed dormancy by directly regulating the expression of *ABSCISIC ACID INSENSITIVE 3* [27], but also functions as a key regulator in the cross talk of salicylic acid (SA) and jasmonic acid (JA) pathways [28]. Another study revealed that GhWRKY41 from *Gossypium hirsutum* enhances osmotic stress tolerance when overexpressed in *Nicotiana benthamiana* [11].

Such studies indicate the breadth of roles of WRKY41 genes from different species, but it is not yet known whether AtWRKY41, and its paralog from *B. napus*, BnWRKY41-1, have similar functions in controlling anthocyanin accumulation.

In the current study, we addressed this question by overexpressing BnWRKY41-1 in *A. thaliana wrky41* mutant lines, using a range of approaches, including RNA sequencing (RNA-Seq) and quantitative real-time PCR (qRT-PCR) analyses, to examine the effects of the transgene in rescuing the mutant phenotypes. Conclusions regarding the similarity of function in influencing anthocyanin production in *A. thaliana* rosette leaves are presented.

## 2. Materials and methods

### 2.1. Plant material and growth condition

The *A. thaliana* ecotype Columbia (Col-0) provided the wild type control for the *wrky41-2* (SALK_028449) mutant in the Col-0 background, which was used in a previous study [27]. The *A. thaliana* plants were grown in an environmental controlled growth chamber under long-day conditions, as described previously [29]. The *B. napus* genotype, Westar, was grown in the greenhouse of South Campus, Northwest A&F University, China. The presence of the *wrky41-2* mutation was confirmed at the DNA and RNA levels using specific primers (Supplementary Table S1).

### 2.2. Gene cloning of BnWRKY41-1 from B. napus

Specific primers were designed to amplify the *BnWRKY41-1* gene according to the full length complementary DNA (cDNA) of *BnWRKY41-1* (GenBank Number XP_013686534.1). RNA was extracted from young leaves of the *B. napus* genotype, Westar, and used to synthesize template cDNA. The PCR amplified *BnWRKY41-1* cDNA was cloned into the pMD19-T vector (TaKaRa Bio, Dalian, China) and the inserts of ten randomly selected single colonies were sequenced by TSINGKE Biological Technology (Beijing, China). Primers for cloning are listed in Supplementary Table S1.

### 2.3. DNA and protein sequence analysis

Nucleotide and amino acid sequences were analyzed using DNASTAR software (DNAStar Co., Madison, USA) and sequences were aligned using MUSCLE software (http://www.ebi.ac.uk/Tools/msa/muscle/). The conserved WRKY domain of BnWRKY41-1 was predicted using a domain search program at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). Phylogenetic analysis was used to investigate the evolutionary relationships among the WRKY41 protein sequences. A minimum evolution tree was generated using MEG6 (http://www.megasoftware.net/), based on a majority-rule consensus from 1000 bootstrap replicates.

### 2.4. Plasmid construction

To construct the *35S:BnWRKY41-1–6HA* vector the PCR amplified *BnWRKY41-1* cDNA, which contained the open reading frame (ORF) without the stop codon, was digested with the restriction endonucleases *Xma*I and *Spe*I and then cloned into the pGreen–35S–6HA vector to obtain the fusion BnWRKY41-1–6HA, under the control of the CaMV35S promoter (*35S:BnWRKY41-1–6HA*). Similarly, the digested PCR fragment was also cloned into pGreen–35S–green fluorescent protein (GFP) to obtain a fusion of BnWRKY41-1–GFP under the control of the CaMV35S promoter (*35S:BnWRKY41-1–GFP*). Primers for plasmid construction are listed in Supplementary Table S1.

### 2.5. Subcellular localization of BnWRKY41-1–GFP fusion protein

The construct of *35S:BnWRKY41-1–GFP* was transiently expressed in young leaves of *N. benthamiana* using a previously reported technique [30]. In brief, the recombinant plasmid *35S:BnWRKY41-1–GFP* was transformed into *Agrobacterium tumefaciens* strain GV3101. After cell culture overnight at 28 °C, *A. tumefaciens* was harvested by centrifugation and resuspended in infiltration liquid media (0.15 mM acetosyringone, 10 mM MgCl2, 10 mM MES-KOH, pH 5.6). The images of the fluorescent signal were collected with a confocal laser scanning microscope (ZEISS LSM 700, Germany) 72 h after agroinfiltration.

### 2.6. Creation of A. thaliana transgenic plants

The *A. thaliana wrky41-2* plants were transformed with *A. tumefaciens strain* GV3101 containing the recombinant plasmid *35S:BnWRKY41-1–6HA* using the floral dip method [31]. When true leaves of seedlings grown on soil emerge, Basta treatment was used to select transgenic plants. The *wrky41-2 35S:BnWRKY41-1–6HA* transgenic plants were genotyped by DNA and RNA analyses and selfed through two more generations to generate T3 transgenic progeny.

### 2.7. Anthocyanin measurement

Anthocyanin levels were measured as previously described [32]. Briefly, rosette leaves homogenized thoroughly with a pestle and mortal were cultured in 300 µL of methanol solution containing 1% HCl overnight at 4 °C, and then extracted with an equal volume of chloroform after adding 200 µL of distilled water. The content of anthocyanins was determined by spectrophotometric detection of the aqueous phase (*A530*–0.25 × *A657*) and normalized to the fresh weight. The experiment was performed using three independent biological replicates, with at least three technical replicates for each biological replicate.

### 2.8. RNA-Seq and data analyses

RNA-Seq analysis was performed of the sixth rosette leaves of 18 individual plants for each genotype of the wild type (Col-0) control and *wrky41-2* mutant in one biological replicate, which were grown in different pots arranged randomly. Two independent biological replicates were sequenced from two different plantings. The analysis was performed using the commercial service Gene Denovo (http://www.

genedenovo.com/) following the standard protocols (http://www.genedenovo.com/product/41.html). The Excel add-in for significance analysis of RNA-Seq was used to identify differential expressed genes (DEGs) between the wild type and *wrky41-2*. The DEGs were functionally classified using the 'biological process' category of Arabidopsis Gene Ontology (http://www.geneontology.com). The DEGs with $\log_2$ ratios $\geq 1$ or $\leq -1$ (only GO Slim identifiers with *false discovery rate* (*FDR*) $\leq 0.05$) are listed in Supplementary Tables S2 and S3.

### 2.9. RNA extraction and qRT-PCR analysis

The *A. thaliana* rosette leaves and *B. napus* young leaves were harvested from at least 8 individual plants, and two independent biological replicates from two different plantings were used for the qRT-PCR expression analysis. Three technical replicates for each biological replicate were analyzed. Total RNA was isolated with a TaKaRa miniBest Plant RNA Extraction Kit (TaKaRa Bio, Dalian, China) following the manufacturer's instructions. Samples were the treated with RNase-free DNAse I to digest any remaining genomic DNA. The first-strand cDNA was synthesized in a 20 μL reaction solution containing approximately 2.0 μg total RNA, using PrimerScript™ reverse transcriptase (TaKaRa Bio, Dalian, China) and oligo (dT) 12–18 as a primer. The qRT-PCR reaction used SYBR Green Master Mix (TaKaRa Bio, Dalian, China) and the calculation of relative gene expression levels was performed as reported previously [33]. The ubiquitously expressed genes *AtACTIN7* and *BnACTIN7* were regarded as the internal reference controls.

## 3. Results

### 3.1. Sequence characterization of BnWRKY41-1

To date, two *BnWRKY41* paralogs have been predicted in the genome of *B. napus* cultivar Zhongshuang11 (ZS11): XP_013686534.1 and XP_013695247.1, designated *BnWRKY41-1* and *BnWRKY41-2*, respectively (Fig. 1A). The cDNA of *BnWRKY41-1* (1624 bp) has an ORF of 993 bp, predicted to encode a 330-amino acid protein (https://www.ncbi.nlm.nih.gov/nuccore/923798306). The cloned *BnWRKY41-1* cDNA sequence from the *B. napus* genotype, Westar, is identical to the *BnWRKY41-1* (ZS11) sequence and so the two predicted BnWRKY41-1 protein sequences (Westar and ZS11) are also the same (Fig. 1A). BnWRKY41-1 has one WRKY domain, consisting of a conserved WRKYGQK motif followed by a 48-amino acid zinc finger motif (Fig. 1A). These features are characteristic of the plant-specific family of WRKY transcription factors [3]. *BnWRKY41-1* is located on the C2 subgenome of the *B. napus* cultivar ZS11, and our sequence results indicated that we had cloned *BnWRKY41-1* from the Westar C2 subgenome. As illustrated in Fig. 1A, BnWRKY41-1 (Westar) has 68% sequence identity with AtWRKY41, whereas their corresponding WRKY domains have 92% sequence similarity at the amino acid level.

We performed a phylogenetic analysis to investigate the evolutionary relationships between BnWRKY41-1 and 38 WRKY41 proteins from 31 plant species. As shown in Fig. 1B, BnWRKY41-1 is most related to the four WRKY41 proteins, including RsWRKY41 (XP_018458950.1), BoWRKY41 (XP_013616531.1), BnWRKY41-2 (XP_013695247.1), and BrWRKY41 (XP_009128690.1). The analysis also predicted that BnWRKY41-1 is closely related to AtWRKY41 from *A. thaliana* (Fig. 1B), suggesting that BnWRKY41-1 may have similar functions with AtWRKY41.

### 3.2. Subcellular localization of BnWRKY41-1 protein

To investigate the subcellular localization of BnWRKY41-1, laser scanning confocal microscopy was used to detect the GFP fluorescence signal of the fusion protein BnWRKY41-1–GFP expressed in *N. benthamiana* leaves. As illustrated in Fig. 2, the GFP signal is specifically localized in the nucleus, consistent with BnWRKY41-1 functioning as a transcription factor.

### 3.3. AtWRKY41 represses anthocyanin accumulation in rosette leaves by inhibiting several key genes contributing to anthocyanin biosynthesis

To understand the putative role of AtWRKY41 in anthocyanin accumulation, the T-DNA insertion mutant SALK_028449, which has been previously designated *wrky41-2* [27], was genotyped, and suppression of AtWRKY41 transcript accumulation was confirmed (Supplementary Fig. S1). We then measured anthocyanin levels and observed that as rosette leaves developed, the anthocyanin content increased significantly in the wild type plants (Fig. 3). Notably, the anthocyanin levels in the rosette leaves were always much higher in *wrky41-2* lines than in the wild type plants at 10, 15, and 20 days after germination (DAG) (Fig. 3). These results suggested that AtWRKY41 represses anthocyanin accumulation in *A. thaliana* rosette leaves.

To elucidate the regulatory networks underlying AtWRKY41-mediated anthocyanin biosynthesis in *A. thaliana*, we performed RNA-Seq analysis of the sixth rosette leaves of the wild type and *wrky41-2* plants at 20 DAG. This revealed 133 differentially expressed genes (DEGs) (Supplementary Tables S2 and S3), of which 79 were expressed at higher levels ('up-regulated'; Supplementary Table S2) and 54 at lower levels ('down-regulated; Supplementary Table S3) in the *wrky41-2* rosette leaves. Based on the functional annotations, five of the up-regulated genes, but none of the down-regulated genes, are related to anthocyanin biosynthesis (Table 1; Supplementary Tables S2 and S3). In addition, six, three and two of the down-regulated genes are annotated as being involved in "oil metabolism", 'amino acid and protein', and 'carbohydrate metabolism', respectively (Supplementary Table S3). We noted that the number of down-regulated genes related to the metabolism of oil, amino acids and proteins, and carbohydrates, was more than that of the up-regulated genes in the *wrky41-2* rosette leaves (Supplementary Tables S2 and S3). Notably, 23 (~29%) of the up-regulated genes and 22 (~41%) of the down-regulated genes are categorized as being involved in stress/defence responses (Supplementary Tables S2 and S3). However, the expression of many regulatory and structural genes associated with anthocyanin biosynthesis did not differ between the *wrky41-2* and wild type rosette leaves (Supplementary Table S4).

We then performed qRT-PCR to verify the up-regulation of genes contributing to anthocyanin accumulation in the *wrky41-2* rosette leaves. Consistent with the RNA-Seq results, expression the levels of three regulatory genes, *AtMYB75* (AT1G56650), *AtMYB111* (AT5G49330), and *AtMYBD* (AT1G70000), and two structural genes, *AT1G68440* and *GLUTATHIONE S-TRANSFERASE PHI 12* (*AtGSTF12*, AT5G17220), were significantly higher in *wrky41-2* rosette leaves compared with the wild type plants (Fig. 4; Table 1; Supplementary Table S2). Taken together, these results suggested that AtWRKY41 inhibits anthocyanin accumulation through repression of these regulatory and structural genes in *A. thaliana* rosette leaves.

### 3.4. Overexpression of BnWRKY41-1 in A. thaliana wrky41-2 prevents the abnormally high anthocyanin content of rosette leaves

To further elucidate the function of BnWRKY41-1 in anthocyanin biosynthesis, *BnWRKY41*-1 was constitutively overexpressed in the *wrky41-2* mutant using the construct *35S:BnWRKY41-1–6HA* (Supplementary Fig. 2A). Four selected independent *wrky41-2 35S:BnWRKY41-1–6HA* T3 homozygous transgenic lines were identified by genomic DNA PCR amplification, using vector-specific primers 35S_P/BnWRKY41-1_R (Supplementary Fig. 2B; Supplementary Table S1). Furthermore, the expression of the *BnWRKY41-1* gene in these transgenic plants was detected by qRT-PCR, and was determined to be lowest in the transgenic line *wrky41-2 35S:BnWRKY41-1–6HA#2*, whereas its expression was not measured in the wild type or *wrky41-2* plants (Supplementary Fig. 2C). Ectopic expression of *BnWRKY41-1* in

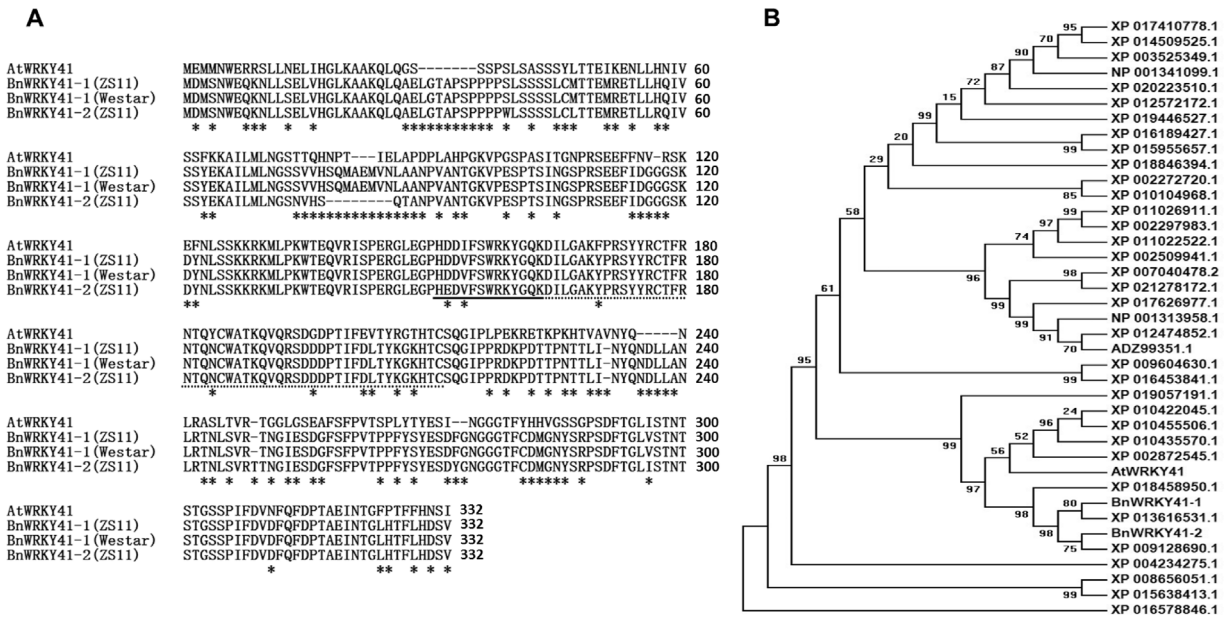


**Fig. 1.** Protein sequence and phylogenetic analyses of WRKY41 proteins.
(A) Alignment of protein sequences of WRKY41 from *Arabidopsis thaliana* and *Brassica napus*, performed using MUSCLE (http://www.ebi.ac.uk/Tools/msa/muscle/). Non-conserved amino acids are indicated by asterisks. The WRKY domain (151-211) is defined by the conserved amino acid sequence WRKYGQK at its N-terminal end, indicated by the solid black line, together with a novel zinc-finger-like motif labeled with dash black lines, as predicted using Conserved Domain Search (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi).
(B) Phylogenetic analysis of 39 WRKY41 proteins from 31 plant species. A neighbor-joining tree (Jones-Taylor-Thornton model) was generated using MEGA6. A bootstrap analysis with 1000 replicates was performed to assess the statistical reliability of the tree topology. The accession numbers corresponding to the species names are as follows: XP_017410778.1 (*Vigna angularis*); XP_014509525.1 (*V. radiata var. radiata*); XP_003525349.1 (*Glycine max*); NP_001341099.1 (*G. max*); XP_020223510.1 (*Cajanus cajan*); XP_012572172.1 (*Cicer arietinum*); XP_019446527.1 (*Lupinus angustifolius*); XP_016189427.1 (*Arachis ipaensis*); XP_015955657.1 (*A. duranensis*); XP_018846394.1 (*Juglans regia*); XP_002272720.1 (*Vitis vinifera*); XP_010422045.1 (*Camelina sativa*); XP_011026911.1 (*Populus euphratica*); XP_002297983.1 (*P. trichocarpa*); XP_011022522.1 (*P. euphratica*); XP_002509941.1 (*Ricinus communis*); XP_007040478.2 (*Theobroma cacao*); XP_021278172.1 (*Herrania umbratica*); XP_017626977.1 (*Gossypium arboreum*); NP_001313958.1 (*G. hirsutum*); XP_012474852.1 (*G. raimondii*); ADZ99351.1 (*G. hirsutum*); XP_009604630.1 (*Nicotiana tomentosiformis*); XP_016453841.1 (*N. tabacum*); XP_019057191 (*Tarenaya hassleriana*); XP_010422045.1 (*Camelina sativa*); XP_010455506.1 (*C. sativa*); XP_010435570.1 (*C. sativa*); XP_002872545.1 (*Arabidopsis lyrata subsp. lyrata*); NP_192845.1 (*Arabidopsis thaliana, AtWRKY41*); XP_018458950.1 (*Raphanus sativus*); XP_013686534.1 (*Brassica napus ZS11, BnWRKY41-1*); XP_013616531.1 (*Brassica oleracea var. oleracea*); XP_013695247.1 (*Brassica napus ZS11, BnWRKY41-2*); XP_009128690.1 (*Brassica rapa*); XP_004234275.1 (*Solanum lycopersicum*); XP_008656051.1 (*Zea mays*); XP_015638413.1 (*Oryza sativa Japonica Group*); XP_016578846.1 (*Capsicum annuum*).

the *wrky41-2* background prevented the elevated anthocyanin content that is typical in *wrky41-2* rosette leaves (Fig. 3). However, the anthocyanin content of rosette leaves in the *wrky41-2 35S:BnWRKY41-1–6HA#2* plants was not lowest among these four transgenic lines (Fig. 3), which suggested that BnWRKY41-1 regulates anthocyanin accumulation in a dose-independent manner when overexpressed in *A. thaliana*.

To investigate how BnWRKY41-1 controls anthocyanin biosynthesis, we measured the expression of genes regulating anthocyanin accumulation in the sixth rosette leaves of *wrky41-2 35S:BnWRKY41-1–6HA* transgenic lines, the expression levels of which are normally elevated in the *wrky41-2* rosette leaves (Fig. 4; Table 1; Supplementary Table S2). We found that the introduction of *35S:BnWRKY41-1–6HA* into the *wrky41-2* mutant restored the abundance of *AtMYB111* and *AT1G68440* transcripts to wild type levels, and indeed the expression of *AtMYB75*, *AtMYBD*, and *AtGSTF12* was somewhat higher than in the wild type plants (Fig. 4). These results suggested that *BnWRKY41-1*, when overexpressed in *A. thaliana*, has a similar role with AtWRKY41 in regulating anthocyanin biosynthesis in rosette leaves.

## 4. Discussion

Anthocyanin biosynthesis in flowers and fruits is characterized in more detail than in vegetative plant parts [34]. Previous studies have shown that the transcription factor, WRKY41, functions as a regulator of seed dormancy [27], plant hormone pathways [28], as well as responses to biotic and abiotic stresses [11,28]. However, the respective roles of WRKY41 genes from *A. thaliana* and its close relative *B. napus*, an important oil-producing crop, in the regulation of anthocyanin accumulation are not well understood. In this study, we demonstrated that BnWRKY41-1 exhibits a similar function with AtWRKY41 in the regulation of anthocyanin biosynthesis in rosette leaves when overexpressed in *A. thaliana*.

It was previously reported that, among vegetative organs in *A. thaliana*, the expression of *AtWRKY41* was highest in leaves [27]. We observed that the *wrky41* mutation resulted in a significant increase in the amount of anthocyanins in *A. thaliana* rosette leaves (Fig. 3). RNA-Seq and qRT-PCR analyses showed that two structural genes (*AT1G68440* and *AtGSTF12*) and three regulatory genes (*AtMYB75*, *AtMYB111*, and *AtMYBD*) were considerably up-regulated in the sixth rosette leaves of *wrky41-2* plants at 20 DAG (Fig. 4; Table 1; Supplementary Table S2). A computational analysis suggested that *AT1G68440*, one of the structural genes, is involved in phenylpropanoid metabolism [35], which leads to the production of *p*-coumaroyl CoA [36], a key substrate for anthocyanin biosynthesis [36,37]. AtGSTF12 is localized in the nucleus cytoplasm, and tonoplast, and functions as a carrier to transport anthocyanins from the cytosol to the tonoplast, thereby promoting anthocyanin accumulation [38–40]. In *A. thaliana*, transcriptional regulation serves as an essential factor for anthocyanin biosynthesis [41,42] and several studies have demonstrated that AtMYB75 strongly enhances anthocyanin accumulation by altering the expression of the structural genes, such as *CHALCONE SYNTHASE* (*AtCHS*), *FLAVONONE 3-HYDROXYLASE* (*AtF3H*), *FLAVONOID 3′-HYDROXYLASE* (*AtF3′H*), *DIHYDROFLAVONOL 4-REDUCTASE* (*AtDFR*), and *ANTHOCYANIDIN SYNTHASE* (*AtANS*), in *A. thaliana* [41,43]. AtMYB111 promotes flavonoid accumulation by activating early anthocyanin biosynthetic genes, including *PHENYLALANINE AMMONIA-LYASE* (*AtPAL*), *AtCHS*, *CHALCONE FLAVANONE ISOMERASE* (*AtCHI*), and *AtF3H* [44,45]. MYBD, a MYB-like Domain transcription factor, increases anthocyanin accumulation via the direct repression of a

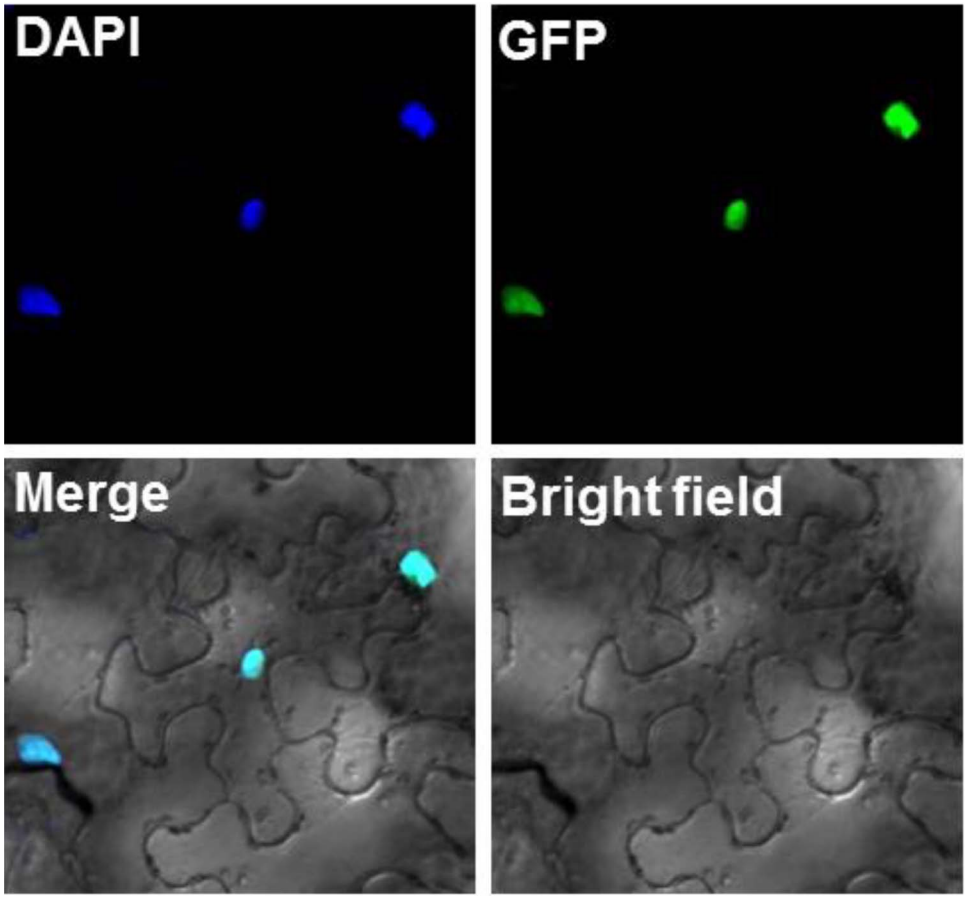


**Fig. 2.** Subcellular localization of BnWRKY41-1 fused with GFP (*35S:BnWRKY41-1–GFP*) and over-expressed in *Nicotiana benthamiana* leaves. DAPI, fluorescence of 4′, 6-diamino-2-phenylindole; Merge, merging of GFP, DAPI, and bright field images.

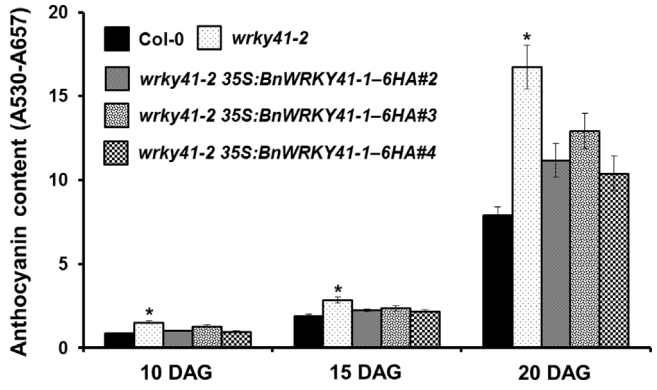


**Fig. 3.** Quantitative assay of anthocyanin content in rosette leaves of the wild type (Col-0), *wrky41-2* and *wrky41-2 35S:BnWRKY41-1–6HA* transgenic plants, 10, 15, and 20 days after germination (DAG). Asterisks indicate significant differences in anthocyanin levels compared to the wild type control (two-tailed paired Student's *t* test, $P \leq 0.05$). Values are means ± SD (n = 3). Error bars denote standard deviations.

**Table 1**
Differentially expressed genes (DEGs) related to anthocyanin accumulation in the sixth true leaves of *wrky41-2* plants, 20 days after germination. DEGs with $|\log_2 \text{ratios}| \geq 1.00$, and only GO Slim IDs with *FDR* ≤ 0.05, are listed here.

| Anthocyanin-related genes | $\log_2$ ratios | Functions | References |
|---|---|---|---|
| *AtMYB75* (AT1G56650) | 2.41 | Promoting anthocyanin accumulation | [41,43] |
| *AtMYB111* (AT5G49330) | 1.36 | Promoting anthocyanin accumulation | [44,45] |
| *AtMYBD* (AT1G70000) | 1.44 | Promoting anthocyanin accumulation | [46] |
| *AT1G68440* | 1.01 | Involved in phenylpropanoid metabolism | [35] |
| *AtGSTF12* (AT5G17220) | 2.35 | Promoting anthocyanin accumulation | [38–40] |

putative MYB family transcription factor, *MYB-LIKE 2* (*AtMYBL2*), which functions as a supressor, and the indirect activation of several structural genes, such as *AtDFR*, *AtANS*, and *UDP-GLUCOSE:-FLAVONOID 3-O-GLUCOSYLTRANSFERASE* (A*tUF3GT*) [46]. We propose that the elevated expression of *AT1G68440*, *AtGSTF12*, *AtMYB75*, *AtMYB111*, and *AtMYBD* (Fig. 4; Table 1; Supplementary Table S2) in *wrky41-2* causes the higher anthocyanin accumulation in *wrky41-2* rosette leaves (Fig. 3). We observed no differences in the transcript levels of the regulatory gene, *AtMYBL2*, or the structural genes, such *AtPAL*, *AtCHS*, *AtCHI*, *AtF3H*, *AtF3′H*, *AtDFR*, and *AtANS*, in *wrky41-2* rosette leaves compared with the wild type rosette leaves (Supplementary Table S4). These genes regulated by at least one of the three elevated regulatory genes, *AtMYB75*, *AtMYB111*, and *AtMYBD* (Fig. 3), are likely regulated by unknown regulatory genes or repressed by AtWRKY41, but the underlying regulatory mechanism has yet to be determined. A previous study indicated that the regulatory gene, *AtABI3*, is directly repressed by AtWRKY41 in maturing and imbibed seeds [26]. However, we observed that its expression was not altered in *wrky41-2* rosette leaves (Supplementary Tables S4), suggesting that AtWRKY4-mediated gene expression may be tissue specific. Notably, 45 (~34%) of the stress/defense-responsive genes accounted for the largest proportion of all DEGs in *wrky41-2* rosette leaves (Supplementary Tables S2 and S3), consistent with AtWRKY41 serving as a key regulator in response to environmental stresses [11,27,28]. Previous studies have demonstrated that anthocyanins function as important molecules in vegetative tissues to enhance tolerance of environmental stresses [47–51]. AtWRKY41 activates and suppresses SA and JA signal transductions, respectively

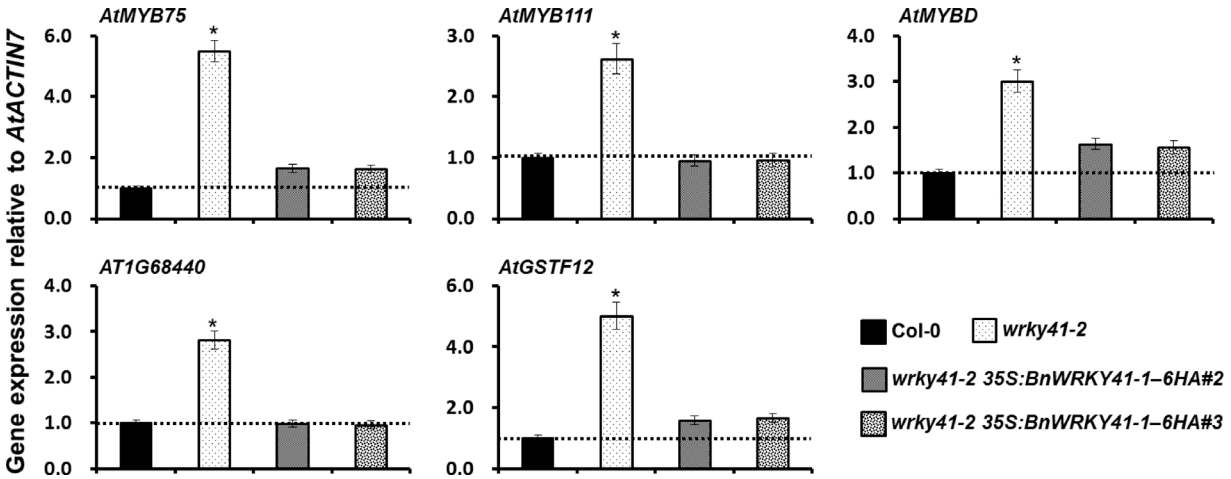


**Fig. 4.** Expression of *AtMYB75*, *AtMYB111*, *AtMYBD*, *AtAT1G68440*, and *AtGSTF12* in the sixth rosette leaves of the wild type (Col-0), *wrky41-2*, and *wrky41-2 35S:BnWRKY41-1–6HA* plants, 20 days after germination, as measured by qRT-PCR. Expression levels were normalized to the expression of the internal reference gene, *AtACTIN7*, and the transcript levels are relative to the wild type, which was set to 1.0. Values are the means of three replicates carried out using cDNA dilutions obtained from two independent RNA extractions. Error bars indicate standard deviations. Asterisks indicate statistically significant differences in gene expression between wild type and *wrky41-2* plants (two-tailed paired Student's *t* test, $P \leq 0.05$).

[28]. Thus, it is possible that these differentially expressed stress/defense-responsive genes (Supplementary Tables S2 and S3) may be directly regulated by AtWRKY41 itself and/or indirectly regulated by the higher anthocyanin accumulation (Fig. 3) and altered SA- and JA-pathways [28] in the *wrky41-2* mutant. These interesting questions need to be further investigated.

Ectopic expression of *BnWRKY41-1*, derived from the C2 subgenome of the *B. napus* genotype Westar, in the *wrky41-2* mutant not only restored wild type levels of anthocyanins (Fig. 3), but also resulted in higher expression levels of several genes that regulate anthocyanin biosynthesis (Fig. 4). These results indicate that the role of BnWRKY41-1 is similar to that of AtWRKY41 in regulating anthocyanin accumulation. The somewhat higher expression level of *AtMYB75*, *AtMYBD*, and *AtGSTF12* in *wrky41-2 35S:BnWRKY41-1–6HA* rosette leaves than in wild type may explain the higher levels of anthocyanins in the transgenic plants (Figs. 3 and 4). BnWRKY41-1 is closely related to BnWRKY41-2 (Fig. 1B), and overexpression of *BnWRKY41-1* did not fully counter the higher anthocyanin content in *wrky41-2* rosette leaves (Fig. 3), suggesting that at least one of the *BnWRKY41* paralogs in the *B. napus* genome, probably *BnWRKY41-2*, may promote anthocyanin accumulation in rosette leaves when expressed in *A. thaliana*.

*B. napus* is the result of an interspecific hybridization of *B. oleracea* and *B. rapa* [52,53] and there has been considerable chromosomal rearrangement, fusion, and deletion in the *B. napus* genome during evolution [54]. We found that the cloned BnWRKY41-1 (Westar) from the *B. napus* genotype Westar has the same predicted protein sequence as BnWRKY41-1 (ZS11, XP_013686534.1) from the *B. napus* cultivar ZS11 (Fig. 1A). This suggests that the function of BnWRKY41 has been conserved during the evolution and divergence of the Westar and ZS11 genotypes.

In summary, we conclude that a WRKY transcription factor, AtWRKY41, represses anthocyanin accumulation in *A. thaliana* rosette leaves by controlling the expression of three regulatory genes (*AtMYB75*, *AtMYB111*, and *AtMYBD*) and two structural genes (*AT1G68440* and *AtGSTF12*) involved in anthocyanin biosynthesis (Figs. 3 and 4; Table 1; Supplementary Table S2). In addition, we determined that the function of BnWRKY41-1 is similar to that of AtWRKY41 in controlling anthocyanin accumulation when overexpressed in *A. thaliana*. In this regard, BnWRKY41-1 is a promising potential target to genetically manipulate *B. napus* and other oil-producing plants to increase the abundance of anthocyanins in rosette leaves.

## Contributions

Shaowei Duan, Jianjun Wang, and Mingxun Chen conceived the project and designed the experiment plans; Shaowei Duan, Jianjun Wang, Chenhao Gao, Changyu Jin, Dong Li, Danshuai Peng, Guomei Du, and Yiqian Li conducted the experiments; Shaowei Duan, Jianjun Wang, and Mingxun Chen analyzed the data and wrote the article with contributions from all the authors.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.plantsci.2017.12.010.

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