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Fagopyrum tataricum FtWD40 Functions as a Positive Regulator of Anthocyanin Biosynthesis in Transgenic Tobacco

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Abstract Tartary buckwheat (*Fagopyrum tataricum* Gaertn.) contains high concentration of flavonoids, which are mainly represented by rutin, anthocyanins, and proanthocyanidins. WD40 transcription factors (TFs) play significant roles in the transcriptional regulation of the anthocyanin biosynthetic pathway. In this study, a WD40-repeat protein gene (designated as *FtWD40*) was identified and characterized from tartary buckwheat. The bioinformatics analyses showed that the putative FtWD40 shared a high level of similarity with MtWD40-1, which is a positive regulator in anthocyanin biosynthesis of *Medicago truncatula*. The yeast one-hybrid assay indicated that FtWD40 had transcriptional activation activities. During florescence, *FtWD40* was highly expressed in flowers compared to other organs. Furthermore, its overexpression in tobacco resulted in a remarkable deepening of petal pigmentation in flowers due to a significant increase in anthocyanins accumulation. Meanwhile, the expression of dihydroflavonol-4-reductase (*DFR*) and anthocyanin synthase (*ANS*) was upregulated 1.95- and 1.56-fold, respectively. In contrast, the expression level was lower for flavonol synthase (*FLS*) in the transgenic lines. To the best of our knowledge, this is the first functional characterization of a WD40 transcription factor (*FtWD40*) from tartary buckwheat that controls the anthocyanin pathway.

Keywords *Fagopyrum tataricum* · Anthocyanin biosynthesis · WD40-repeat protein · Transcription factor · Transgenic tobacco

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Introduction

Tartary buckwheat (*Fagopyrum tataricum* Gaertn), an important nutrient-rich cereal crop with pharmacological values, is mostly grown in Asian countries (Park and others 2011). Tartary buckwheat has been recognized as a health-promoting food, because of its abundant nutrients and high levels of anti-oxidants, including rutin and anthocyanins (Li and others 2015). Anthocyanins, as a kind of flavonoids, have received extensive attention owing to their prospective health benefits, such as anti-mutagenic, antimicrobial, anti-inflammatory, anti-oxidant, and anti-hypertensive properties (Lai and others 2016; Lim and others 2016).

The anthocyanin biosynthetic pathway has been studied in multiple species (Grotewold 2006; Koes and others 2005). Anthocyanin biosynthesis is always in response to multiple stress factors such as light, salt, and temperature (Das and others 2012). Furthermore, the literature has shown that the response is largely attributed to the regulation of anthocyanin-related gene expression through transcription factors involving MYB, bHLH, and WD40 (Grotewold 2006; Lepiniec and others 2006; Lin-Wang and others 2010). The three transcription factors generally activate anthocyanin biosynthesis as a MBW complex, in which the WD40 protein usually stabilizes the complex through binding to the bHLH, and

interacts with MYB in the complex (Hichri and others 2011). In addition, WD40 also can independently bind to its DNA targets related to anthocyanin biosynthesis (Dong and others 2014). TRANSPARENT TESTA GLABRA1 (TTG1), which belongs to the WD40-repeat protein family in *Arabidopsis thaliana*, has been identified to effectively promote anthocyanin accumulation by increasing the expression of *ANS* and *DFR* (Mehrtens and others 2005). Ectopic expression of the grapevine gene *WDR1* enhances anthocyanin accumulation in shoots and rosette leaves, compared to wild-type plants in *Arabidopsis* (Matus and others 2010). In other cases, WD40 interacts with bHLHs or MYBs, and regulates the expression of anthocyanin-related genes as a crucial part of the MBW. In *Arabidopsis*, TTG1 interacts with both MYB TF TT2 and bHLH TF TT8 (Baudry and others 2004). However, in some other species, the WD40 protein has been shown to only interact with bHLH, but not with MYB (Dubos and others 2008; Grotewold and others 2000). Yeast two-hybrid (Y2H) and Bimolecular Fluorescence Complementation (BiFC) assays demonstrated that the apple WD40 protein MdTTG1 interacts with bHLH but not MYB proteins to regulate anthocyanin accumulation (An and others 2012). So, it is necessary to clone more WD40 genes, and characterize their function in the anthocyanins biosynthetic pathway of plants.

Recent studies of TFs in tartary buckwheat have mainly focused on MYBs and bHLHs. In our previous studies, FtMYB1/2 were found to control proanthocyanidin biosynthesis (Bai and others 2014). *FtMYB3* inhibited anthocyanin synthesis in transgenic tobacco plants by decreasing the expression of chalcone isomerase (*CHI*), dihydroflavonol 4-reductase (*DFR*), and anthocyanidin synthase (*ANS*). In addition, FtbHLH1 was involved in anthocyanin biosynthesis of tartary buckwheat (unpublished). However, WD40 transcript factor is unknown in tartary buckwheat so far. To further study the mechanism of the single transcription factor or MBW complex in anthocyanin biosynthesis of tartary buckwheat, we turned our attention to the WD40 transcription factor family. In this study, a WD40-type gene, *FtWD40*, was firstly isolated and characterized from tartary buckwheat. We evaluated the expression profiles of the gene in tartary buckwheat. The results of stable transformation experiments in *Nicotiana tabacum* strongly indicated that *FtWD40* plays a significant role in the anthocyanin biosynthesis pathway in tartary buckwheat. Possible mechanisms of how *FtWD40* exerts biological effect in tobacco are discussed as well as its possible importance for plant metabolic engineering.

Materials and Methods

Plant Materials and Growth Conditions

About 500 seeds of tartary buckwheat seeds ("Xiqiao No.2") were sown in the experimental field of Sichuan Agriculture University, Ya'an Sichuan, China, and the germination rate was above 90%. The tender roots, stems, leaves, and flowers were harvested separately during the florescence stage at 6 pm, and each sample was derived from 3 plants. All samples were frozen in liquid nitrogen immediately and kept at −80 °C for further use. Three independent biological replicates were measured for each sample. To detect the transcription level of the *FtWD40* gene under different abiotic stress treatments, tartary buckwheat seeds were germinated in a growth chamber at 25 °C and approximately 60% humidity for 48 h in the dark and then grown in 1/2 Hoagland's solution liquid medium with a 16 h light/8 h dark cycle at 25/22 °C (day/night) for two weeks. Wild-type and T1 transgenic tobacco (*Nicotiana tabacum* T12) were germinated in a growth chamber at 25 °C and approximately 60% humidity (16 h light/8 h dark).

Abiotic Stress Treatments

To examine whether *FtWD40* expression is mediated by any external stresses, *FtWD40* transcription levels in tartary buckwheat were examined after exposure to abiotic stresses that are known to promote anthocyanin accumulation (Shin and others 2016). Two-week-old tartary buckwheat seedlings were treated with the following conditions: 150 mM NaCl, 30% PEG-6000, 100 μM abscisic acid (ABA), 1 mM salicylic acid (SA), UV-B light (302 nm, 0.1 mW/cm^2), and 4 °C (Su and others 2014; Zhou and others 2015), separately. For all the treatments, the seedlings (3 plants) were collected at 0, 6, 12, 24, and 48 h (0 h was non-treated control) and immediately frozen in liquid nitrogen for total RNA extraction. Three independent biological replicates were measured for each sample.

Cloning of FtWD40

The RNA was extracted from the various plant organs using an RNAout kit (Tiandz, China), and cDNA was synthesized with a RevertAid First Strand cDNA Synthesis kit (MBI, USA). The cDNA of flowers was used as a PCR template to amplify the gene of *FtWD40* from tartary buckwheat. A pair of degenerate primers, WD40-S and WD40-A, was designed according to the conserved region of known *WD40* genes from other plants, such as *Arabidopsis thaliana* and *maize*. To get the full-length cDNA sequence of *FtWD40*, rapid-amplification of cDNA ends (RACE)

was performed using a Smart Race cDNA Amplification kit (Takara, Japan). Primers were used to obtain both ends of the *FtWD40* gene as follow: F5-GSP, F5-NGSP1, and F5-NGSP2 for the 5′primer, and F3-GSP, F3-NGSP1, and F3-NGSP2 for the 3′primer. The Open Reading Frame (ORF) sequence of *FtWD40* was obtained with primers WD40-F and WD40-R. The PCR products were subcloned into the pMD@19-T simple vector (Takara, Japan) and sequenced. All primer sequences are listed in Table S1.

Molecular Characterization of FtWD40

The sequence analysis of the *FtWD40* gene was conducted using BLASTx and BLASTn programs in the National Center of Biotechnology Information (NCBI) database. The nucleotide and amino acid sequences were aligned using the Clustal X software. Phylogenetic analysis was performed using MEGA (Version 5).

Transactivation Assay

The transcription activation assay was performed in the yeast strain AH109 with *LacZ* and *His* reporter genes. The ORF sequence of the *FtWD40* was ligated into the yeast expression vector pBridge (Biovector Inc., USA) after double digestion with *EcoR*I and *BamH*I. The pBridge empty plasmids and AH109 cells was used as a negative controls, and pBridge-GmMYBJ6 (Yang and others 2009) served as a positive control. The resulting plasmids were transformed into yeast AH109 cells using a lithium acetate-mediated method (Gietz and others 1992). The yeast transformants were verified by colony PCR and plated on the SD/-His-Trp medium for 3 d at 30 °C. Transcription activation was evaluated according to the growth status of yeast cells and 5-bromo-4-chloro-3-indolyl-β-D-galacto-pyranoside (X-gal). The primers (WDf, WDr) used in vector construction are listed in Table S3.

Stable Transformation in Tobacco

The plasmids used in the stable expression assay were constructed by ligating the ORF sequence of *FtWD40* with pCAMBIA1301 using *Kpn*I and *BamH*I. The recombinant plasmids and the pCambia1301 vector (as an internal control) were transformed into *Nicotiana tabacum* (T12) by utilizing the *Agrobacterium*-mediated leaf disk transformation method (Horsch and others 1985). Transgenic plants were cultured on Murashige and Skoog (MS) medium and screened in 1/2 MS medium containing hygromycin (50 mg L^{-1}, w/v). Positive lines were selected and verified by PCR. The primers used in vector construction are listed in TableS3.

Analysis and Determination of Total Anthocyanin Content in Plant Organs

All fresh samples, including transgenic tobaccos and tartary buckwheat, were homogenized in liquid nitrogen and analyzed according to the reported method (Rabino and Mancineli 1986). 1 mL of acidic methanol (1% HCl, v/v) was added to 200 mg of fresh plant material. The samples were incubated for 18 h at room temperature under moderate shaking. The extracts were diluted with an equal volume of water, and then an equal volume of chloroform was added to the extract. The samples were vortexed gently for a few seconds followed by centrifugation for 5 min at 15,000×*g*. The aqueous phase was used for the determination of absorbance at 530 nm and 657 nm. The anthocyanins were quantified using the following equation: $Q_{Anthocyanins}=(A_{530} - 0.25 \times A_{657}) \times M^{-1}$, where $Q_{Anthocyanins}$ is the amount of anthocyanin, A_{530} and A_{657} are the absorptions at the indicated wavelengths, and M is the weight of the plant material used for extraction [g]. Three independent biological replicates were measured for each sample.

Quantitative Real-Time PCR (qRT-PCR) Analysis

The qRT-PCR analysis was performed to confirm the *FtWD40* expression in tartary buckwheat under abiotic stresses and to analyze the expressions of anthocyanin-related genes in transgenic tobacco. Total RNA was isolated using the RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA) from samples. The RNA was then converted into first-strand cDNA using ReverTra Ace (Toyobo, Osaka, Japan) with a random hexamer. Transcription levels of *FtWD40* and six anthocyanin-related genes, including phenylalanine ammonialyase (*PAL*, GenBank ID: AB289452), chalcone synthase (*CHS*, GenBank ID: AF311783), anthocyanidin synthase (*ANS*, GenBank ID: EB427369), flavonoid3′-hydroxylase (*F3′H*, GenBank ID: AB289449), dihydroflavonol 4-reductase (*DFR*, GenBank ID:EF421429), and flavonol synthase (*FLS*, GenBank ID: AB289451), were analyzed using qRT-PCR. The primers for qRT-PCR were designed by the Primer Premier 5 software based on obtained *FtWD40* and other gene cDNA sequences (Table S2). The gene *FtH3* (Histone 3, GenBank ID: HM628903) and *β-actin* (GenBank ID: AB158612) were used as the reference genes in tartary buckwheat and tobacco, respectively. qRT-PCR was performed with a SYBR Premix EX Taq Kit (TaKaRa, Japan) in a total reaction volume of 20 µL, which contained 10 µL of SYBR Green Mix, 0.5 µM of each primer, and 1 µL of cDNA. The reactions were carried out in a CFX96 Real-Time PCR thermocycler (Bio Rad, USA) according to the manufacturer's protocol. The PCR protocol was as follows: 95 °C for 5 min, followed by 39 cycles of 95 °C for 15 s, 60 °C

for 15 s, and 72 °C for 20 s. The data were evaluated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001). Three independent biological replicates were measured for each sample.

Statistical Analyses

Three independent biological replicates were measured for each sample and the data presented as the mean ± standard error (SE). Where applicable, data were analyzed by Student's t test in a two-tailed analysis. Values of $P<0.05$ or <0.01 were considered to be statistically significant.

Results

Cloning of FtWD40 from *F. tataricum*

Our aim was to isolate WD40 transcription factors (TFs) that may be involved in flavonoid biosynthesis in *F. tataricum*. Using homology cloning and RACE technology, full-length cDNA sequences of *FtWD40* (1097 bp, GenBank ID: KX059426) were characterized from flowers of *F. tataricum*, which contained a 1035 bp ORF and encoded a protein of 344 amino acids. Sequence alignments with the NCBI database showed the *FtWD40* protein was a member of the WD40 family.

Molecular Characterization of FtWD40

The amino acid sequence of FtWD40 was aligned with previously reported WD40 proteins. The majority of the homology was observed within the WD40 repeat motifs, normally a 40-amino acid tandem repeat characterized by Gly-His (GH) and Trp-Asp (WD) doublet residues (Van Nocker and Ludwig 2003). The four hypothetical WD40 repeat motifs were found in the predicted amino acid sequence of FtWD40 (Fig. 1).

A phylogenetic tree was drawn to establish the biological functions and evolutionary relationships between FtWD40 and other known WD40s (Fig. 2). The dendrogram was classified into two clusters, the PALE ALEURONE COLOR1 (PAC1) and the MP1 clades. Mapping experiments have positioned Mp1 to the long arm of chromosome 5 close to Pac1. Analysis of the Mp1 sequences revealed a lack of a nuclear localization signal, suggesting that it might encode a cytosolic protein. Meanwhile, the Pac1 was involved in anthocyanin accumulation (Hernandez and others 2000). Phylogenetic analysis showed that FtWD40 is highly homologous to AtTTG1, PfWD40, PhAN11, and VvWDR1, which contribute to regulating anthocyanins.

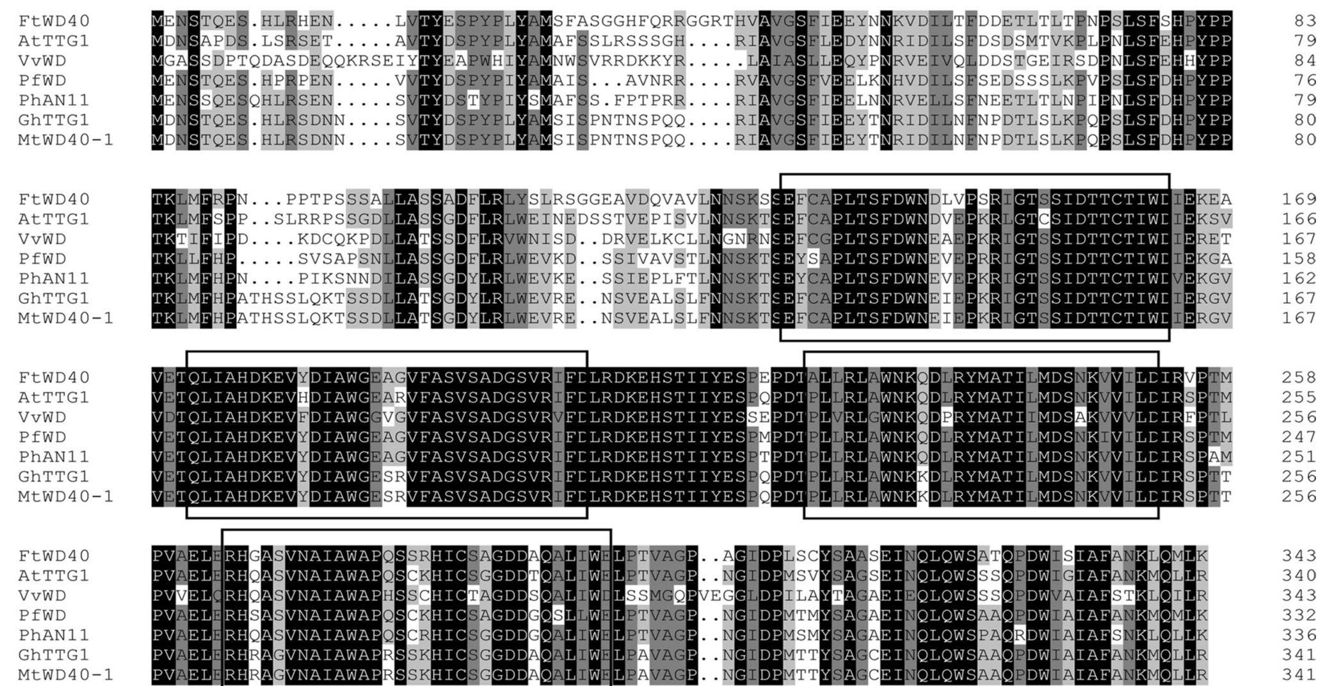

Fig. 1 Alignment of FtWD40 with related WD40 proteins by Clustal X: AtTTG1 (NM_122360) from *Arabidopsis thaliana*, VvWD (NP_001268006) from *Vitis vinifera*, PfWD (BAB58883) from *Perilla frutescens*, PhAN11 (AAC18914) from *Petunia x hybrida*, GhTTG1 (AAM95641) from *Gossypium hirsutum*, and MtWD40-1 (ABW08112) from *Medicago truncatula*. The WD40 conserved domains were indicated with black boxes

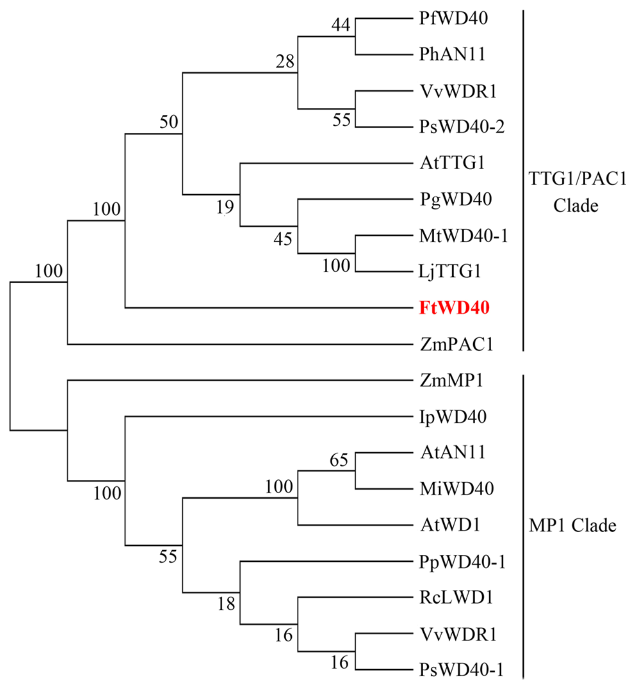

Fig. 2 Phylogenetic relationships between FtWD40 and WD40s from *Fagopyrum tataricum* and other plant species. The GenBank accession numbers are as follows: PfWD40 (BAB58883) from *Perilla frutescens*, PhAN11 (AAC18914) from *Petunia x hybrida*, VvWDR1 (ABF66625) from *Vitis vinifera*, PsWD40-2 (AIU98520) from *Paeonia suffruticosa*, AtTTG1 (NP_197840) from *Arabidopsis thaliana*, PgWD40 (ADV40946) from *Punica granatum*, MtWD40-1 (ABW08112) from *Medicago truncatula*, LjTTG1 (BAH28880) from *Lotus japonicus*, ZmPAC1 (AAM76742) from *Zea mays*, ZmMP1 (AAR01949) from *Zea mays*, IpWD40 (BAE94397) from *Ipomoea purpurea*, AtAN11 (AAC18912) from *Arabidopsis thaliana*, MiWD40 (CAE76645) from *Matthiola incana*, AtWD1 (NP_172751) from *Arabidopsis thaliana*, PpWD40-1 (ADN52336) from *Pyrus pyrifolia*, RcLWD1 (XP_002512788) from *Ricinus communis*, VvWDR2 (NP_001268006) from *Vitis vinifera*, PsWD40-1 (AIU98519) from *Paeonia suffruticosa*. FtWD40 were highlighted in *red*. (Color figure online)

Expression Pattern of FtWD40 in Various Tissues

To verify whether there is a relationship between *FtWD40* expression and anthocyanin accumulation in different tissues, the expression pattern of *FtWD40* was analyzed in four organs of tartary buckwheat using qRT-PCR. Tissues, including roots, stems, leaves, and flowers, were harvested separately from the whole mature plants. During florescence, the expression level of *FtWD40* was the highest in flowers, moderate in roots, and lowest in leaves and stems. The level of *FtWD40* transcription in flowers was 1.2-, 2.1-, and 1.9-fold higher than that in roots, stems, and leaves, respectively (Fig. 3a). In addition, the anthocyanin content analysis showed a similar profile and the expression of *FtWD40* in various tissues (Fig. 3b).

Transcriptional Analysis of the FtWD40 Gene Under Different Stresses

The result of qRT-PCR showed substantial differences of *FtWD40* transcript abundance in response to multiple stresses except SA treatment (Fig. 4). Under 4 °C and NaCl stresses, the transcription of *FtWD40* continuously increased after 12 h and reached the maximum level at 24 to 48 h. For UV-B and PEG-6000 treatment, *FtWD40* expression showed no obvious change in 12h, but a remarkable increase was detected from 24 h. For ABA treatment, the highest activation was detected after 12 h of treatment. SA stress had no obvious effect on the transcription of *FtWD40*. On the whole, NaCl, ABA, and low temperature had more pronounced effects on *FtWD40* expression than other treatments.

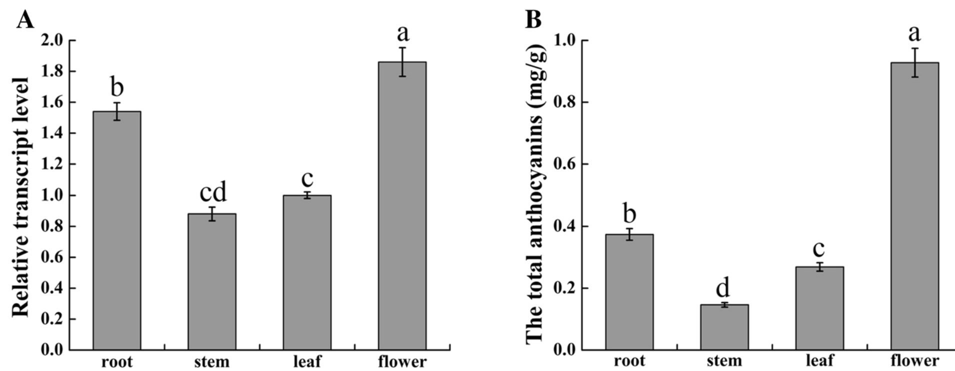

Fig. 3 **a** Relative expression level of *FtWD40*; **b** Total anthocyanin accumulation in different tissues. *FtH3* was used as an internal control. The expression data were normalized to leaf (set at 1) using the comparative threshold cycle method. *Error bars* represent the standard deviation of triplicate runs for qRT-PCR. The values shown with different lowercase letters within different organs are significantly different (Tukey test: $P < 0.05$)

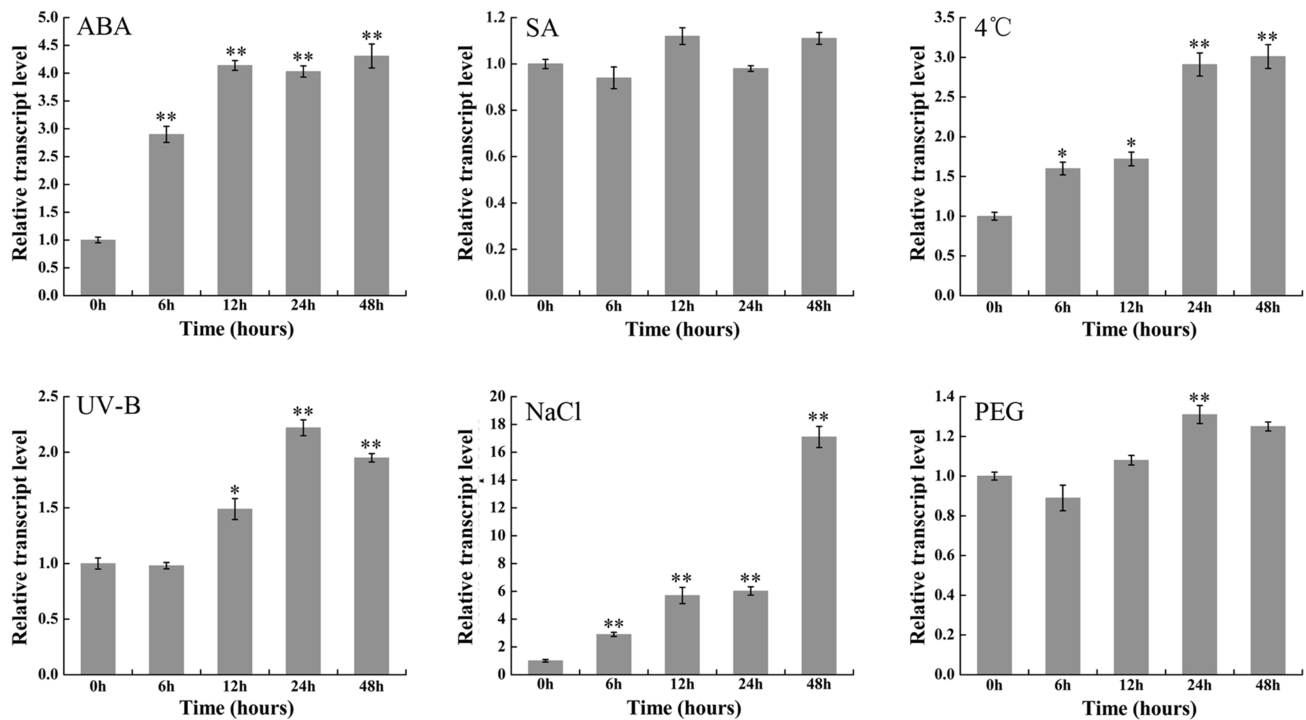

Fig. 4 Expression analysis of *FtWD40* gene in tartary buckwheat seedlings under ABA, SA, 4 °C, UV-B, NaCl, and drought treatments. The mRNA expression patterns were examined with a qRT-PCR assay. The $2^{-\Delta\Delta CT}$ method was used to determine the relative expression, and the expression levels of genes in 0 h (no treated) were set to "1". *FtH3* was used as a housekeeping gene. Each value represents the mean of three replicates, and *error bars* indicate standard deviations (± SD). $^{*}P<0.05$ and $^{**}P<0.01$ indicate significant differences between the control and stressed tartary buckwheat

Transcription Activation of FtWD40

The transcriptional activity of FtWD40 was analyzed using a yeast assay system. The ORF of *FtWD40* was cloned into the pBridge vector and then transformed into yeast AH109. Yeast cells containing pBridge-FtWD40 and pBridge-GmMYBJ6 grew well in SD/-Trp/-His medium, whereas cells containing pBridge did not grow (Fig. 5a). Meanwhile, there was no color change in the negative control, and the positive control and

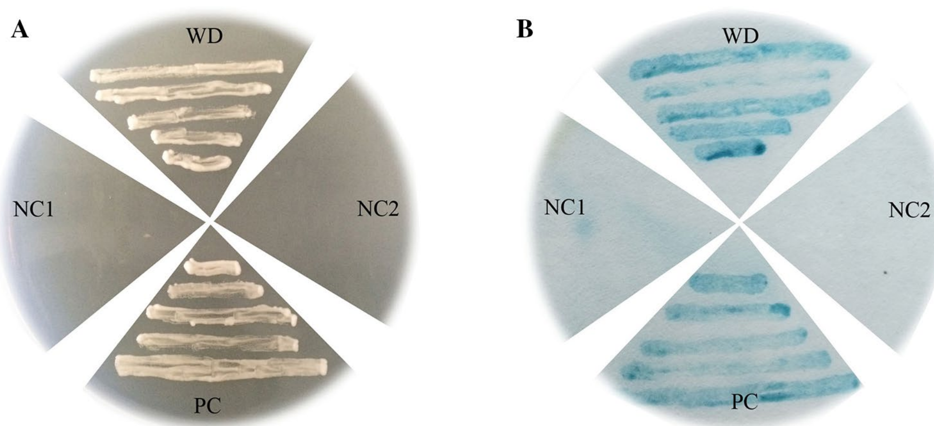

Fig. 5 Transactivation analysis of FtWD40 in yeast. **a** Before the galactosidase filter lift assay. **b** After the galactosidase filter lift assay. WD: pBridge-FtWD40; PC (Positive Control): pBridge-G*m*MYBJ6; NC1 (Negative Control): pBridge pasmid ; NC2 (Negative Control): AH109. Fusion proteins of pBridge-FtWD40, pBridge-GmMYBJ6 and pBridge were expressed in yeast strain AH109. The transformants were streaked on the SD/Trp^{-}/His^{-} medium. The plates were incubated for 3 d and subjected to X-gal assay

pBridge-FtWD40 had a significant blue reaction in the presence of X-gal (Fig. 5b).

Identification of Transgenic Tobacco Lines

The ORF of *FtWD40* was ectopically expressed in tobacco using the binary vector pCAMBIA1301-*FtWD40* (Fig. 6a). Seven independent transgenic tobacco lines that overexpressed the *FtWD40* gene were obtained from Hyg-resistance selection and were cultured under the same conditions (Supplemental Fig. 1). PCR analysis confirmed the presence of the transformed *FtWD40* gene in all transgenic lines, and the absence of endogenous *FtWD40* in wild-type (WT) and control check (CK, plant was transformed with empty pCAMBIA1301 vector) tobacco plants (Fig. 6b). The qRT-PCR analysis showed that the expression level of *FtWD40* was significantly higher in the transgenic plants, especially #2, #5, and #7, than in CK (Fig. 6c). Therefore, the three lines were used for further experiments.

Ectopic Expression of FtWD40 Enhances Anthocyanin Accumulation in Transgenic Tobacco

Interestingly, there is no substantial difference in morphology between the transgenic plants and wild-type. However, transgenic tobacco lines harboring *FtWD40* were affected in flower color, with a remarkable deepening of petal pigmentation compared with the CK tobacco (Fig. 7a). This change in the corolla color of transgenic tobacco was already visible prior to anthesis. To confirm that the deeper flower color was attributed from an increased pigment levels synthesized from the anthocyanin pathway, the total anthocyanin was determined. In the transgenic tobacco lines #2, #5, and #7, anthocyanin contents were improved to 3.38-, 2.85-, and 3.25-fold, respectively, as compared

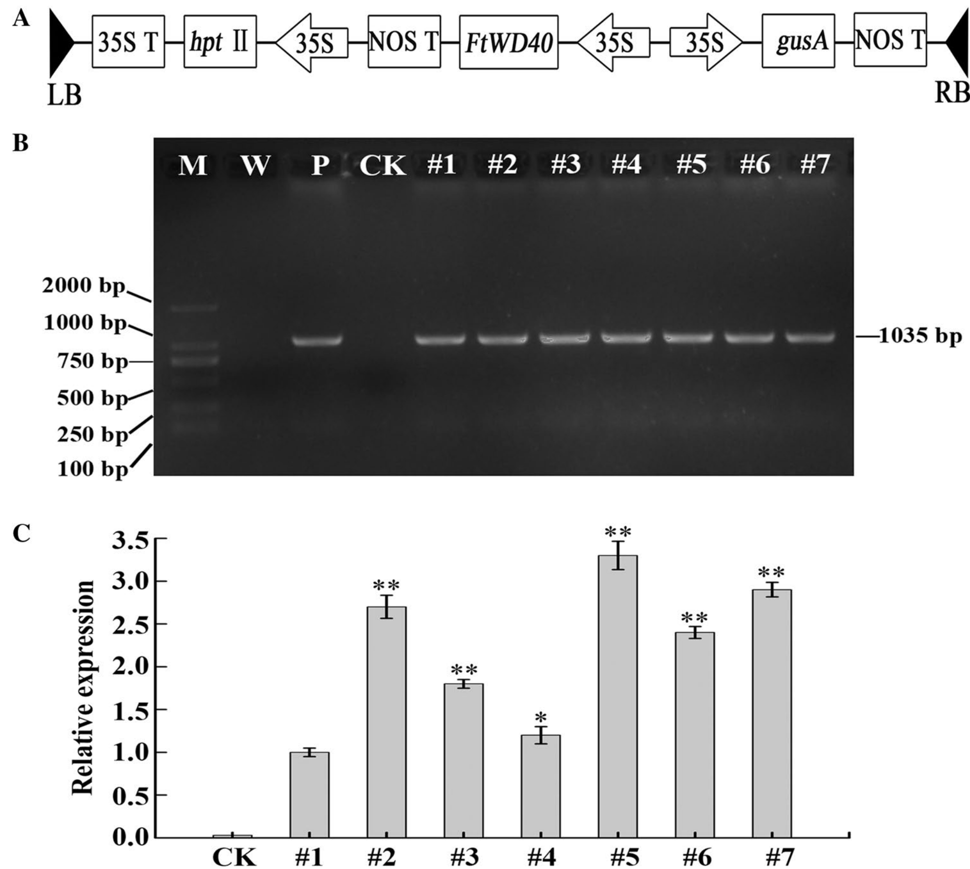

Fig. 6 Molecular analyses of the *FtWD40*-overexpressing tobacco plants. **a** Schematic diagram of the T-DNA region of the binary plasmid pCAMBIA1301-*FtWD40*. *LB* left border; 35S T, CaMV 35S terminator; *hpt II*, hygromycin phosphotransferase II gene; 35S, cauliflower mosaic virus (CaMV) 35S promoter; *NOS T*, nopaline synthase terminator; *FtWD40*, tartary buckwheat WD40 transcription factor gene; *gusA*, *β*-glucuronidase gene; *RB*, right border. **b** PCR analysis of transgenic plants. *Lane M* DL2000 DNA marker; *Lane W* water as negative control; *Lane P* plasmid pCAMBIA1301-*FtWD40* as positive control; *Lane CK* wild-type; *Lanes #1-#7* transgenic plants. **c** Expression analysis of the *FtWD40* gene in transgenic tobacco plants and CK. The tobacco *Ntaction* gene was used as an internal control. Data are presented as mean ± SD (n = 3). * and ** indicate a significant difference from that of WT at $P < 0.05$ and <0.01, respectively, by Student's *t* test

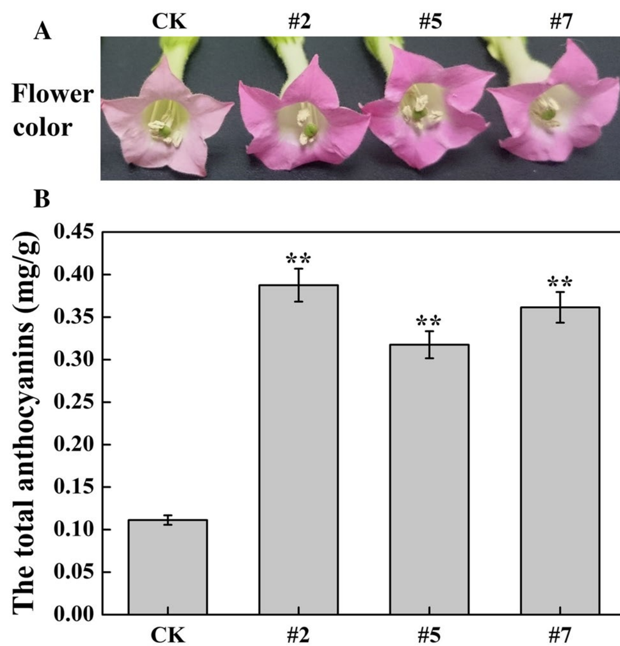

Fig. 7 **a** Floral phenotypes of transgenic tobacco plants expressing tartary buckwheat *FtWD40*. **b** Quantification of total anthocyanins in transgenic tobacco seedling leaves. The CK plant was transformed with empty pCambia1301 vector. #-n (n represents the *line* number) transgenic lines were transformed with pCambia1301-*FtWD40* vector. Each value represents the mean of three replicates, and *error bars* indicate standard deviations (±SD). ** indicate a significant difference from that of CK at $p < 0.01$, by Student's t-test

to the CK (Fig. 7b). Notably, the expression of *FtWD40* in transgenic tobacco correlated with the pigmentation enhancement observed in the petals.

Expression of Genes Involved in Anthocyanin Biosynthesis in Transgenic Tobacco

To determine whether the gene expression could potentially account for the remarkable increase of pigmentation in flowers, the expression levels of anthocyanin-related genes were evaluated using qRT-PCR with *NtActin* as an internal control. It showed that these genes can be grouped into three categories in terms of their responsiveness to *FtWD40* (Fig. 8). The first category, phenylalanine ammonialyase (*PAL*), chalcone synthase (*CHS*) and flavonoid 3′-hydroxylase (*F3′H*), showed an inconspicuous correlation to the *FtWD40* expression. The expression of the three genes was similar to the CK. The effect of the constitutive *FtWD40* expression was more pronounced for dihydroflavonol 4-reductase (*DFR*) and anthocyanidin synthase (*ANS*). A distinct increase (approximately 95%) in the transcript of *DFR* was observed and, to a lesser extent, a slighter increase (approximately 56%) in the latter gene. The single member of the third category is the flavonol synthase (*FLS*), whose expression was markedly repressed. The data indicated that only two genes (*DFR* and *ANS*) from the lower end of the flavonoid pathway, more directly related to the biosynthesis of anthocyanins, were dramatically *FtWD40* responsive, and the alteration of *DFR* was most drastic.

Discussion

In this study, we isolated *FtWD*40 encoding a WD-repeat protein that is possibly involved in the regulation of anthocyanin biosynthesis. The amino acid sequence of FtWD40 showed a high identity with other proteins from dicotyledonous plants, such as PhAN11, GhTTG1, AtTTG1, and PFWD (Table S4). Through analyzing the FtWD40 amino acid sequence, we observed the presence of four predicted WD repeats that are remarkably well conserved among plants, which suggested that FtWD40 is a highly conserved protein in the process of evolution. In addition, we detected a relationship between anthocyanin accumulation and *FtWD40* expression. *FtWD40* was ubiquitously expressed in all tissues examined, and the expression level was significantly correlated with the anthocyanin content in various organs. These results suggested that *FtWD40* might be involved in the metabolism of anthocyanins in tartary buckwheat as a transcriptional activator. The majority of the WD40 genes were responsive to abiotic stresses such as salinity, drought, and low temperature. BnSWD1, which is a novel WD40 repeat-containing protein, was upregulated after treatment with abscisic acid, salicylic acid, and methyl jasmonate (Lee and others 2010). Similarly, *FtWD40* was highly sensitive to abiotic stresses, suggesting that it had a protective role via regulation of anthocyanin biosynthesis (Dong and others 2014). Indeed, tartary buckwheat benefits from the anthocyanin accumulation, which protects seedlings from UV radiation, temperature change, and drought (Tsurunaga and others 2013). The phylogenetic tree placed FtWD40 into dicotyledon subgroups involved in anthocyanin biosynthesis. So, we propose that FtWD40 is a positive regulator of anthocyanin biosynthesis in tartary buckwheat.

Three major transcription factor families, including MYB, bHLH, and WD40, generally activate anthocyanin biosynthesis as a part of the MBW complex (Espley and others 2007; Lin-Wang and others 2014). It is worth noting that WD40 is an important part of the WD40/MYB/bHLH complex and interacts with bHLHs or MYBs to regulate anthocyanin or proanthocyanidin biosynthesis (Gonzalez and others 2008; Qi and others 2011; Ramsay and Glover 2005; Schaart and others 2013). In *Arabidopsis*, functional analysis of *TTG1*/WDR, *AtMYB123*/MYB, and *AtbHLH42*/bHLH indicated that these three proteins synergistically control anthocyanin accumulation (Mehrtens and others

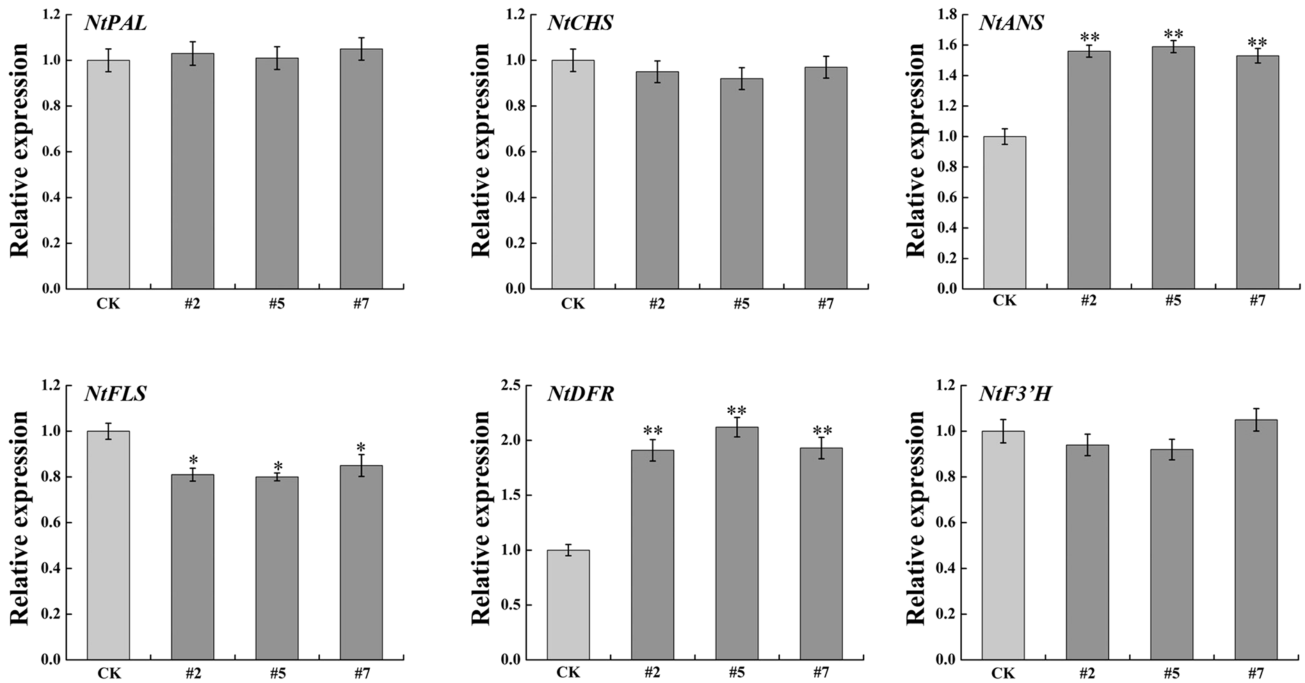

Fig. 8 Analysis of anthocyanin-related gene expression levels in *FtWD40* transgenic tobacco seedling leaves. The transcripts of six anthocyanin biosynthetic genes were detected by qRT-PCR from transgenic lines and compared with control lines. The $2^{-\Delta\Delta CT}$ method was used to determine the relative expression, and the expression levels of genes in CK were set to "1". β-actin was used as a housekeeping gene. Each value represents the mean of three replicates, and *error bars* indicate standard deviations (±SD). The CK plant was transformed with empty pCambia1301 vector. #-n (n represents the *line* number) transgenic lines were transformed with pCambia1301-*FtWD40* vector. $^{*}P<0.05$ and $^{**}P<0.01$ indicate significant differences between control and transgenic tobacco

2005). In petunia, the transcription factors *AN2* and *JAF13* or *AN1* are thought to positively regulate the anthocyanin biosynthetic pathway (Albert and others 2011). Nevertheless, our results showed that overexpression of the *FtWD*40 gene can improve anthocyanin accumulation in *N. tabacum* without co-transferring other factors, which is consistent with *PFWD* from *Perilla frutescens* (Sompornpailin and others 2002). In addition, the overexpression of *IbWD40* increased the expression of the anthocyanin biosynthesis-related gene (Dong and others 2014). Thus, overexpression of *FtWD40* alone could also improve the synthesis of plant anthocyanins.

Furthermore, transcription factors control the synthesis of anthocyanin by regulating the expression of downstream target genes. The expression of early and late biosynthetic genes (EBGs and LBGs, respectively) appears to be regulated separately by different TFs (Dubos and others 2010; Nesi and others 2001). We examined the effect of *FtWD40* on anthocyanin-related genes in transgenic tobacco. Similarly, our result indicated that there was little or no enhancement of the earlier genes. However, *FtWD40* not only upregulated the expression of *NtDFR* and *NtANS* but also reduced the *NtFLS*. It is generally known that *DFR* and *FLS* play an important role in anthocyanin and flavonol biosynthesis; *DFR* and *FLS* compete for the same substrate, dihydroflavonol (Davies and others 2003). This being so, *FtWD40* increased *DFR* expression and suppressed *FLS* expression, which might switch biosynthesis from dihydroflavonol to anthocyanin. When we ectopically expressed *FtWD40* in tobacco, the seedlings of transgenic lines accumulated more anthocyanins than the control plant (Fig. 6b). Simultaneously, the color of the flowers obviously deepened. Our results are similar to those of previous studies, in which overexpression of the proanthocyanidins (PAs) structural gene *ANRs* resulted in a visible decrease in flower color in tobacco (Han and others 2012). However, *PAP1*(MYB) overexpression can affect the transcription of all genes involved in the phenylpropanoid pathway (Tohge and others 2005). Further research is necessary to reveal the differences among different plant species.

Taken together, whether at the transcriptional level or metabolic level, evidence from our transgenic tobacco experiments showed that transcription factor FtWD40 could effectively increase anthocyanin accumulation in a heterogonous host. Because of the health-promoting properties and the economic market for anthocyanins in human health, there is a growing interest in the development of functional foods rich in anthocyanins (Zhang and others 2012). Furthermore, recent research has suggested that transcription factors, which have the potential

ability to regulate multiple structural genes, are more effective compared to single structural genes for plant metabolic engineering (Aharoni and Galili 2011). In this respect, owing to their potential to regulate anthocyanin accumulation, our data may provide new clues and potential opportunities for increasing the commercial value of tartary buckwheat by metabolic engineering of anthocyanins.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

References

Aharoni A, Galili G (2011) Metabolic engineering of the plant primary secondary metabolism interface. Curr Opin. Biotech 22:239–244

Albert NW, Lewis DH, Zhang H, Schwinn KE, Jameson PE, Davies KM (2011) Members of an R2R3 - MYB transcription factor family in *Petunia* are developmentally and environmentally regulated to control complex floral and vegetative pigmentation patterning. Plant J 65:771–784

An XH, Tian Y, Chen KQ, Wang XF, Hao YJ (2012) The apple WD40 protein MdTTG1 interacts with bHLH but not MYB proteins to regulate anthocyanin accumulation. J Plant Physiol 169:710–717

Bai YC, Li CL, Zhang JW, Li SJ, Luo XP, Yao HP, Chen H, Zhao HX, Park SU, Wu Q (2014) Characterization of two tartary buckwheat R2R3-MYB transcription factors and their regulation of proanthocyanidin biosynthesis. Physiol Plantarum 152:431–440

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

Das PK, Shin DH, Choi S-B, Park Y-I (2012) Sugar-hormone cross-talk in anthocyanin biosynthesis. Mol Cells 34:501–507

Davies KM, Schwinn KE, Deroles SC, Manson DG, Lewis DH, Bloor SJ, Bradley JM (2003) Enhancing anthocyanin production by altering competition for substrate between flavonol synthase and dihydroflavonol 4-reductase. Euphytica 131:259–268

Dong W, Niu L, Gu J, Gao F (2014) Isolation of a WD40-repeat gene regulating anthocyanin biosynthesis in storage roots of purple-fleshed sweet potato. Acta Physiol Plant 36:1123–1132

Dubos C, Le Gourrierec J, Baudry A, Huep G, Lanet E, Debeaujon I, Routaboul JM, Alboresi A, Weisshaar B, Lepiniec L (2008) MYBL2 is a new regulator of flavonoid biosynthesis in *Arabidopsis thaliana*. Plant J 55:940–953

Dubos C, Stracke R, Grotewold E, Weisshaar B, Martin C, Lepiniec L (2010) MYB transcription factors in *Arabidopsis*. Trends Plant Sci 15:573–581

Espley RV, Hellens RP, Putterill J, Stevenson DE, Kutty-Amma S, Allan AC (2007) Red colouration in apple fruit is due to the activity of the MYB transcription factor, MdMYB10. Plant J 49:414–427

Gietz D, St Jean A, Woods RA, Schiestl RH (1992) Improved method for high efficiency transformation of intact yeast cells. Nucleic Acids Res 20:1425

Gonzalez A, Zhao M, Leavitt JM, Lloyd AM (2008) Regulation of the anthocyanin biosynthetic pathway by the TTG1/bHLH/Myb transcriptional complex in *Arabidopsis* seedlings. Plant J 53:814–827

Grotewold E (2006) The genetics and biochemistry of floral pigments. Annu Rev Plant Biol 57:761–780

Grotewold E, Sainz MB, Tagliani L, Hernandez JM, Bowen B, Chandler VL (2000) Identification of the residues in the Myb domain of maize C1 that specify the interaction with the bHLH cofactor R. Proc.Natl Acad Sci 97:13579–13584

Han Y, Vimolmangkang S, Soria-Guerra RE, Korban SS (2012) Introduction of apple *ANR* genes into tobacco inhibits expression of both *CHI* and *DFR* genes in flowers, leading to loss of anthocyanin. J Exp Bot 65:2437–2447

Hernandez J, Pizzirusso M, Grotewold E (2000) The maize *Mp1* gene encodes a WD-repeat protein similar to *An11* and *TTG*. Maize Genet Coop Newsl 74:24.

Hichri I, Barrieu F, Bogs J, Kappel C, Delrot S, Lauvergeat V (2011) Recent advances in the transcriptional regulation of the flavonoid biosynthetic pathway. J Exp Bot 62:2465–2483

Horsch RB, Fry J, Hoffmann NL, Eichholtz DA, Rogers SG, Fraley RT (1985) A simple and general method for transferring genes into plants. Science 227:1229–1231

Koes R, Verweij W, Quattrocchio F (2005) Flavonoids: a colorful model for the regulation and evolution of biochemical pathways. Trends Plant Sci 10:236–242

Lai B, Du LN, Liu R, Hu B, Su WB, Qin YH, Zhao JT, Wang HC, Hu GB (2016) Two LcbHLH transcription factors interacting with LcMYB1 in regulating late structural genes of anthocyanin biosynthesis in *Nicotiana* and *Litchi chinensis* during anthocyanin accumulation. Front Plant Sci 7:166

Lee S, Lee J, Paek KH, Kwon SY, Cho HS, Kim SJ, Park JM (2010) A novel WD40 protein, BnSWD1, is involved in salt stress in *Brassica napus*. Plant Biotechnol Rep 4:165–172

Lepiniec L, Debeaujon I, Routaboul JM, Baudry A, Pourcel L, Nesi N, Caboche M (2006) Genetics and biochemistry of seed flavonoids. Annu Rev Plant Biol 57:405–430

Li SJ, Bai YC, Li CL, Yao HP, Chen H, Zhao HX, Wu Q (2015) Anthocyanins accumulate in tartary buckwheat (*Fagopyrum tataricum*) sprout in response to cold stress. Acta Physiol Plantarum 37: 159–167

Lim SH, Song JH, Kim DH, Kim JK, Lee JY, Kim YM, Ha SH (2016) Activation of anthocyanin biosynthesis by expression of the radish R2R3-MYB transcription factor gene *RsMYB1*. Plant Cell Rep 35:641–653

Lin-Wang K, Bolitho K, Grafton K, Kortstee A, Karunairetnam S, McGhie TK, Espley RV, Hellens RP, Allan AC (2010) An R2R3 MYB transcription factor associated with regulation of the anthocyanin biosynthetic pathway in *Rosaceae*. BMC Plant Biol 10:50

Lin-Wang K, McGhie TK, Wang M, Liu Y, Warren B, Storey R, Espley RV, Allan AC (2014) Engineering the anthocyanin regulatory complex of strawberry (*Fragaria vesca*). Front Plant Sci 5:651

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods 25:402–408

Matus J, Poupin M, Cañón P, Bordeu E, Alcalde J, Arce-Johnson P (2010) Isolation of *WDR* and *bHLH* genes related to flavonoid synthesis in grapevine (*Vitis vinifera L.*). Plant Mol Biol Rep 72:607–620

Mehrtens F, Kranz H, Bednarek P, Weisshaar B (2005) The *Arabidopsis* transcription factor MYB12 is a flavonol-specific regulator of phenylpropanoid biosynthesis. Plant Physiol 138:1083–1096

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

Park NI, Li X, Suzuki T, Kim SJ, Woo SH, Park CH, Park SU (2011) Differential expression of anthocyanin biosynthetic genes and anthocyanin accumulation in tartary buckwheat cultivars 'Hokkai T8'and 'Hokkai T10'. J Agr Food Chem 59:2356–2361

Qi T, Song S, Ren Q, Wu D, Huang H, Chen Y, Fan M, Peng W, Ren C, Xie D (2011) The Jasmonate-ZIM-domain proteins interact with the WD-Repeat/bHLH/MYB complexes to regulate jasmonate-mediated anthocyanin accumulation and trichome initiation in *Arabidopsis thaliana*. Plant Cell 23:1795–1814

Rabino I, Mancinelli AL (1986) Light, temperature, and anthocyanin production. Plant Physiol 81:922–924

Ramsay NA, Glover BJ (2005) MYB–bHLH–WD40 protein complex and the evolution of cellular diversity. Trends Plant Sci 10:63–70

Schaart JG, Dubos C, Romero De La Fuente I, van Houwelingen AM, de Vos RC, Jonker HH, Xu W, Routaboul JM, Lepiniec L, Bovy AG (2013) Identification and characterization of MYB-bHLH-WD40 regulatory complexes controlling proanthocyanidin biosynthesis in strawberry (*Fragaria x ananassa*) fruits. New Phytol 197:454–467

Shin DH, Choi MG, Kang CS, Park CS, Choi SB, Park YI (2016) A wheat R2R3-MYB protein PURPLE PLANT1 (TaPL1) functions as a positive regulator of anthocyanin biosynthesis. Biochem Bioph Res Co 469:686–691

Sompornpailin K, Makita Y, Yamazaki M, Saito K (2002) A WD-repeat-containing putative regulatory protein in anthocyanin biosynthesis in *Perilla frutescens*. Plant Mol Biol 50:485–495

Su LT, Li JW, Liu DQ, Zhai Y, Zhang HJ, Li XW, Zhang QL, Wang Y, Wang QY (2014) A novel MYB transcription factor, GmMYBJ1, from soybean confers drought and cold tolerance in *Arabidopsis thaliana*. Gene 538:46–55

Tohge T, Nishiyama Y, Hirai MY, Yano M, Nakajima Ji, Awazuhara M, Inoue E, Takahashi H, Goodenowe DB, Kitayama M (2005) Functional genomics by integrated analysis of metabolome and transcriptome of *Arabidopsis* plants over-expressing an MYB transcription factor. Plant J 42:218–235

Tsurunaga Y, Takahashi T, Katsube T, Kudo A, Kuramitsu O, Ishiwata M, Matsumoto S (2013) Effects of UV-B irradiation on the levels of anthocyanin, rutin and radical scavenging activity of buckwheat sprouts. Food Chem 141:552–556

Van Nocker S, Ludwig P (2003) The WD-repeat protein superfamily in *Arabidopsis*: conservation and divergence in structure and function. BMC Genomics 4:50

Yang W, Wu Y, Tang Y (2009) Expressing and functional analysis of *GmMYBJ6* from soybean. Yi Chuan 31:645–653

Zhang ZL, Zhou ML, Tang Y, Li FL, Tang YX, Shao JR, Xue WT, Wu YM (2012) Bioactive compounds in functional buckwheat food. Food Res Int 49:389–395

Zhou M, Wang C, Qi L, Yang X, Sun Z, Tang Y, Tang Y, Shao J, Wu Y (2015) Ectopic expression of *Fagopyrum tataricum* FtMYB12 improves cold tolerance in *Arabidopsis thaliana*. J Plant Growth Regul 34:362–371