Cereal Research Communications 44(1), pp. 35–46 (2016)
DOI: 10.1556/0806.43.2015.030
First published online October 12, 2015

Identification of a Candidate Gene for Rc-D1, a Locus Controlling Red Coleoptile Colour in Wheat

Y.Q. Wang[1,3,4], X.J. Hou[2], B. Zhang[1,3], W.J. Chen[1,3], D.C. Liu[1,3], B.L Liu[1,3]* and H.G. Zhang[1,3]*

[1]Key Laboratory of Adaptation and Evolution of Plateau Biota (AEPB), Northwest Institute of Plateau Biology, Chinese Academy of Sciences, Qinghai Xining 810008, China
[2]College of Biologic and Geographic Sciences, Qinghai Normal University, Qinghai Xining 810008, China
[3]Qinghai Province Key Laboratory of Crop Molecular Breeding, Xining 810008, China
[4]University of Chinese Academy of Sciences, Beijing 100049, China

(Received 12 January 2015; Accepted 20 March 2015)

Red coleoptile is an easily observed agronomic trait of wheat and has been extensively studied. However, the molecular mechanism of this trait has not yet been revealed. In this study, the MYB gene *TaMYB-D1* was isolated from the wheat cultivar 'Gy115', which possesses red coleoptiles. This gene resided at the short arm of the homoelogous group 7 chromosomes. *TaMYB-D1* was the only gene expressed in the coleoptiles of 'Gy115' and was not expressed in 'Opata' and 'CS', which have uncoloured coleoptiles. Phylogenetic analysis placed TaMYB-D1 very close to ZmC1 and other MYB proteins regulating anthocyanin biosynthesis. The encoded protein of *TaMYB-D1* had an integrated DNA binding domain of 102 amino acids and a transcription domain with 42 amino acids, similar to the structure of ZmC1. Transient expression analysis in onion epidermal cells showed that TaMYB-D1 was located at the plant nucleus, which suggested its role as a transcription factor. The expression of *TaMYB-D1* was accompanied with the expression of *TaDFR* and anthocyanin biosynthesis in the development of the coleoptile of 'Gy115'. Transient expression analysis showed that only *TaMYB-D1* induced a few 'Opata' coleoptile cells to synthesize anthocyanin in light, and the gene also induced a colour change to red in many cells with the help of *ZmR*. All of these results suggested *TaMYB-D1* as the candidate gene for the red coleoptile trait of 'Gy115'.

Keywords: *Triticum aestivum,* red coleoptile, anthocyanin biosynthesis, regulatory network

Introduction

The wheat coleoptile is the pointed protective sheath that covers the emerging shoot. Unlike the flag leaves rolled up within, the preemergent coleoptile does not accumulate significant protochlorophyll or carotenoids and is thus generally very pale. Some coleoptiles do, however, accumulate purple anthocyanin pigments. The function of red coleoptiles in the wheat life cycle is unclear, but it has been speculated that they protect the emerging

*Corresponding authors; E-mails: blliu@nwipb.cas.cn; hgzhang@nwipb.ac.cn

shoot from strong light. Because coleoptile colour is easily observed, this trait has been used to describe wheat varieties and has received extensive study.

The anthocyanin metabolic pathway has been determined for many plants. The main structural genes of anthocyanin biosynthesis encode phenylalanine ammonia lyase *(PAL),* chalcone synthase *(CHS),* chalcone isomerase *(CHI)*, favanone 3-hydroxylase *(F3H),* favonoid 3-hydroxylase *(F3'H),* dihydroXavonol 4-reductase *(DFR),* leucoanthocyanidin dioxygenase *(LDOX),* and Xavonoid 3-O-glucosyltransferase *(UFGT)* (Winkel-Shirley 2001). These structural genes are regulated mainly by two major classes of transcription factors: basic helix–loop–helix (bHLH) type and Myb type (Winkel-Shirley 2001). The inactivation of any one could block the whole metabolic pathway, causing a pale phenotype in plant tissue. However, allelic variations of the *MYB* genes more commonly cause colour differentiation in plants. For example, DNA sequence variation within the promoter of functional *VvmybA1* is associated with the flesh pigmentation content of intensely coloured grape varieties (Kobayashi et al. 2005; Porret et al. 2006; This et al. 2007). Similar functional *MYB* regulators have also been observed for *Arabidopsis MYB75 (PAP1)* and *AtMYB90 (PAP2)* (Borevitz et al. 2000), petunia *AN2* (Quattrocchio et al. 1999), sweet potato *MYB1* (Mano et al. 2007), legume *LAP1* (Peel et al. 2009), apple *MdMYBA* and *MdMYB1* (Ban et al. 2007; Takos et al. 2006), and *Epimedium sagittatum EsMYBA1* (Huang et al. 2013). In monocotyledons, the famous *MYB* regulator *ZmC1,* related to the discovery of transposons, regulates anthocyanin biosynthesis in maize (McClintock 1950). A 19-bp deletion in the coding sequence of *TaMYB10-B* is responsible for the white grain trait in the wheat cultivars 'Chinese Spring' (CS), 'Nor67', and 'Norin17'. However, these three genes have not been linked with the red coleoptile trait (Himi et al. 2004), leaving the key gene for this trait in wheat unknown.

The anthocyanin pigmentation of wheat coleoptiles is controlled by *Rc* genes (*Rc1*, *Rc2*, and *Rc3*, on the short arms of chromosomes 7A, 7B, and 7D, respectively) based on classic genetics (Khlestkina et al. 2002). Wheat cultivars carrying any one of these three genes show red coleoptiles. For example, the wheat cultivar 'Hope' (*Rc1Rc2rc3*) shows intense coleoptile anthocyanin pigmentation, while 'CS' (*rc1rc2rc3*) shows no pigmentation (uncoloured coleoptiles) (Himi et al. 2005). The substitution lines 'CS' ('Hope' 7A) *(Rc1rc2rc3),* 'CS' ('Hope' 7B) (*rc1Rc2rc3*), and 'CS' (*Aegilops tauschii* 7D) *(rc1rc2Rc3)* also show red coleoptiles (Himi et al. 2011; Khlestkina et al. 2008). Through analyzing the expression of structural genes in the coleoptiles of the above substitution lines, each dominant *Rc* allele has been hypothesized to encode a transcription activator (Himi et al. 2011; Khlestkina et al. 2008). Using ZmC1 as a probe, the MYB gene has been located in the wheat chromosomes 7A, 7B, 7D, 5A, 4B, 4D, and one of these six loci has been mapped on chromosome 7D in position similar to that known for the Rc-D1 gene (Li et al. 1999). However, bHLH transcription activators have not been detected on the short arms of chromosomes 7A, 7B, and 7D (Shoeva 2014). This result indicated that a homolog of C1 could be the candidate gene for Rc in bread wheat.

In the present study, the isolation and functional analysis of a MYB transcript factor on the short arm of the 7D chromosomes were performed to determine the key gene controlling the red coleoptile trait.

Materials and Methods

Plant materials

The common wheat (*T. aestivum* L.*,* 2n = 6x = 42, AABBDD) cultivars 'CS', 'Opata', and 'Gy115' were used in this study. 'Gy115' possesses red coleoptiles, while 'Opata' and 'CS' possess uncoloured coleoptiles. Previous research has shown, using 84 recombinant inbred lines, that the red coleoptile of 'Gy115' is controlled by one dominant gene differing from 'Opata' (Liu 2009). 'CS' nullitetrasomic lines (N7AT7B, N7BT7A, and N7DT7B) were used to locate the sites of the gene on the chromosomes.

Genomic DNA, total RNA, and cDNA preparation

DNA was isolated from 1 g of 10-day-old seedlings according to the methods of Yan et al. (2002). Total RNA was extracted from approximately 0.5 g of coleoptiles with the Tiangen RNAprep Pure Plant Kit (Tiangen, China). cDNA was obtained from total RNA using the Thermo RevertAid First Strand cDNA Synthesis Kit (Invitrogen, USA).

Gene isolation

The coding sequences of ZmC1 (AF320613) relative to anthocyanin in maize were blasted against the genomic sequence database of common wheat (AABBDD) (https://urgi.versailles.inra.fr/blast/blast.php) (International Wheat Genome Sequencing Consortium 2014). Three contigs very similar to ZmC1 were present at the short arm of the homoeologous group 7 chromosomes: gnl_IWGSC_7AS_IWGSC_chr7AS_ab_k71_contigs_longerthan_200_4108742, gnl_IWGSC_7BS_IWGSC_chr7BS_ab_k71_contigs_longerthan_200_3084267, and gnl_IWGSC_7DS_IWGSC_chr7DS_ab_k71_contigs_longerthan_200_2723852. Two similar contigs, scaffold101589 and caffold43518, were found in the *T. urartu* (progenitor of the wheat A genome) (http://gigadb.org/dataset/100050) (Ling et al. 2013) and *Ae. tauschii* (progenitor of the wheat D genome) (http://gigadb.org/dataset/100054) (Jia et al. 2013) genomic databases, respectively. After alignment, conserved primers covering the start and stop codons were designed to isolate the *MYB* genes at the short arm of the homoeologous group 7 chromosomes.

PCR amplifications were conducted using high-fidelity Phusion DNA polymerase (Thermo-Fisher Scientific, USA) in the GeneAmp PCR System 9700 (Applied Biosystems, USA) and employed the following procedure: 2 min of denaturation at 98 °C; 35 cycles of 15 s at 98 °C, 30 s at 61 °C, and 30 s at 72 °C; and a final extension of 5 min at 72 °C. The PCR products were extracted from the 1.0% agarose gel using the Tiangen TIANgel Midi Purification Kit (Tiangen, China) and were cloned into the pGEM-T Easy Vector plasmid (Promega Corporation, USA). The recombinant plasmid was then transformed into DH5α *Escherichia coli* cells, and sixty positive clones were sent to a commercial company (Huada Gene, China) for sequencing. All primers used in this study are listed in supplement table 1.

Bioinformatic analysis

The sequence alignments were conducted using Vector NTI 10 software (Invitrogen, USA). The primers were designed using Primer5 software (Premier Biosoft, Canada). The phylogenetic trees of the MYB proteins were constructed using MEGA 4.0 (Tamura et al. 2007). The gene structures were drawn using Gene Structure Display Server (http://gsds.cbi.pku.edu.cn/) (Guo et al. 2007).

Subcellular location

The vector pk7FWG2-R, which contains the fusion *GFP* (green fluorescent protein) gene driven by the 35S promoter, was used for subcellular targeting in this study. The coding region of the *TaMYB-D1* gene was inserted into the pk7FWG2-R vector by a Gateway Cloning System (Invitrogen, USA). The vector pk7FWG2-R-TaMYB-D1 and control vector pk7FWG2-R were delivered into onion epidermal cells by particle bombardment according to the method described by von Arnim (2007). GFP expression in the onion epidermal cells was observed and photographed using a Leica Tcs Sp2 confocal laser scanning microscope (Leica, Germany) after 18 h of culturing at 25 °C.

Transient expression

The pBract214 transient vector, which contains the maize ubiquitin promoter, was used for the transient expression assay. The transient plasmids pBract214-TaMYB-D1, pBract214-ZmR (bHLH gene in maize), and pBract214-ZmC1 (MYB gene in maize) were constructed using the Gateway Cloning Kit (Invitrogen, USA). The plasmids were delivered into the coleoptiles of 'Opata' by particle bombardment according to the methods of Ahmed et al. (2003). Four plasmids were delivered: pBract214-ZmR, pBract214-ZmC1, pBract214-TaMYB-D1, and pBract-control. Additionally, pBract214-TaMYB-D1 and pBract214-ZmC1 were mixed with pBract214-ZmR for transient expression. As a positive control, pBRACT214-GUS was bombarded at 100 ng/gun in all treatments. All treated coleoptiles were observed and photographed using a stereoscope produced by Leica Co., Germany. The red cells were counted, and the cell numbers were calculated using Microsoft Excel 2003. Finally, these coleoptiles were placed into GUS dye overnight to detect the GUS protein (von Arnim 2007).

Expression profiles

The coleoptiles were collected at 1-day intervals from 2 to 6 days after germination and prepared based on the procedures of Himi et al. (2005). The coleoptiles in the light treatment were grown under a light incubator (light intensity 100 μmol s per m^2, 23 °C, 16/8 h), while those in the dark treatment were grown in a dark incubator (23 °C). RT-PCR (reverse transcription PCR) was performed with the primers TaMYB-D1-F and TaMYB-D1-R to examine the expression level of *TaMYB-D1*, and the primers Tubulin-F

and Tubulin-R were used for the *tubulin* gene to standardize the cDNA template amount. The expression of *TaDFR* was also measured to represent that of structural genes in the anthocyanin biosynthesis pathway.

Results

Isolation of TaMYB-D1

Amplification products were obtained using the conserved primers TaMYB-D1-F and TaMYB-D1-R in the genomes of 'Gy115', 'Opata', and 'CS'. These products were almost the same sizes as those revealed using agarose gel (Fig. 1A). After sequencing, three genes could be recovered from the 'Gy115' product, while only one gene was obtained from both the 'Opata' and 'CS' products. The objective segment targeted by the conserved primers could not be obtained in the N7AT7B 'CS' nullitetrasomic line but was present in N7BT7A, N7DT7A, and 'CS' (Fig. 1). Therefore, the gene in 'CS' and 'Opata' was deduced as located on the 7A chromosome, and *TaMYB-A1* was designated as this gene (Accession number: KP136430). The other two genes in 'Gy115' were not present in the 'CS' genome, and their chromosomes could not be located with the nullitetrasomic lines. One of these genes was the same as the contig caffold43518 downloaded from the *Ae. tauschii* genomic database and was therefore designated as *TaMYB-D1* (Accession number: KP136432). The other gene was designated as *TaMYB-B1* (Accession number: KP136431). Interestingly, only *TaMYB-D1* was expressed in the coleoptiles of 'Gy115', while none of the genes were expressed in the coleoptiles of 'Opata' and 'CS' (Fig. 1B). Therefore, the functional analysis focused on *TaMYB-D1*.

Bioinformatic analysis

TaMYB-D1 was assigned to the same branch as *AtMYB12*, *AtTT2*, *ZmC1*, *TaMYB10-A*, *TaMYB10-B*, *TaMYB10-D*, *TaMYB3*, and *TaMYB320* in the phylogenetic tree (Fig. 2A).

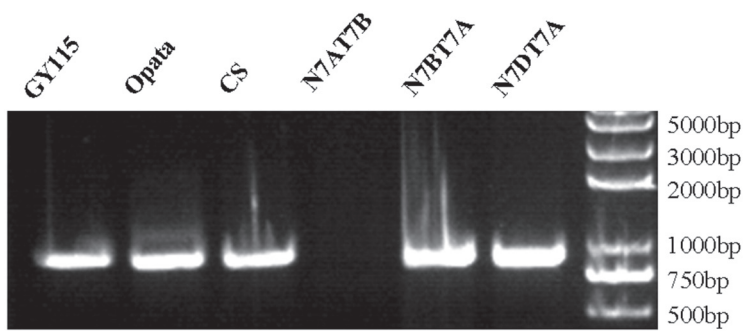

Figure 1. Amplification products using conserved primers TaMYB-D1-F and TaMYB-D1-R. A, the templates were the genomic DNA of Gy115, Opata, CS, N7AT7B, N7BT7A and N7DT7A. Gy115, Opata, CS were bread wheat cultivars. N7AT7B, N7BT7A and N7DT7A were 'CS' nullitetrasomic lines. B, the templates were the cDNAs from the coleoptiles of Gy115, Opata and CS three days after germination

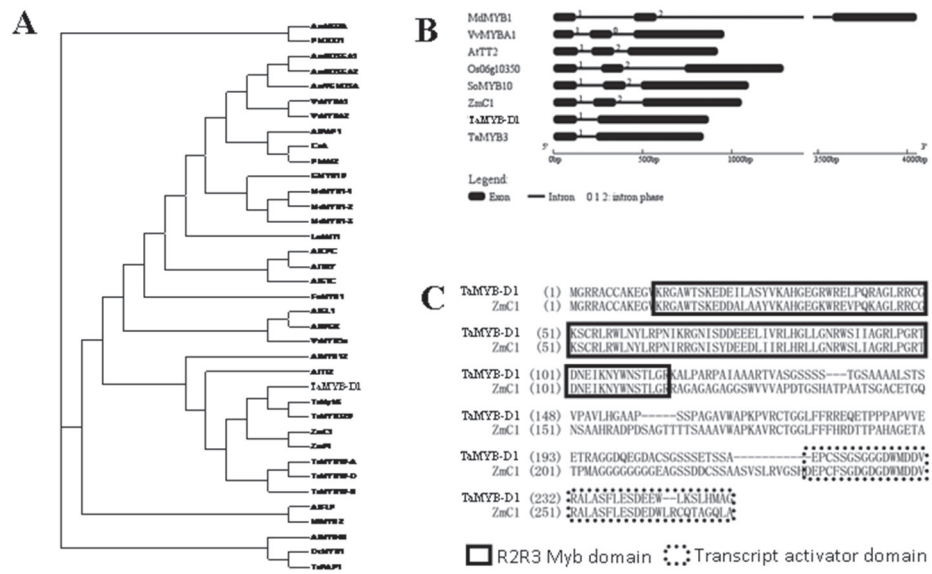

Figure 2. Bioinformatic analysis of TaMYB-D1. A, phylogenetic tree of MYB proteins based on MYB domains (AmMIXTA, CAA55725; AmROSEA1, ABB83826; AmROSEA2, ABB83827; AmVENOSA, ABB83828; AtCPC, NP_182164; AtETC, NP_171645; AtFLP, NP_563948; AtGL1, AAC97387; AtMYB12, ABB03913; AtMYB61, NP_172425; AtPAP1, ABB03879; AtTRY, NP_200132; AtTT2, Q9FJA2; AtWER, AAF18939; CaA, CAE75745; DcMYB1, BAE54312; FaMYB1, AAK84064; GMYB10, CAD87010; LeANT1, AAQ55181; MdMYB1-1, ABK58136; MdMYB1-2, ABK58137; MdMYB1-3, ABK58138; NtMYB2, BAA88222; PhAN2, AAF66727; PhODO1, AAV98200; VvMYB5a, AAS68190; VvMYBA1, BAD18977; VvMYBA2, BAD18978; ZmC1, AAA33482; and ZmPl, AAA19821; TaMYB-D1, KP136432). B, gene structures of partial MYB genes. (*VvMYBA1*, AB111101; *ZmC1*, AF320613; *MdMYB1*, DQ222406; AtTT2, NC_003076; *VvMYB5a*, NC_012014.3; and *AtTRIPTYCHON*, NC_003076.8; *TaMYB-D1*, KP136432). Black boxes represent exons, and lines represent introns. C, sequence alignment of ZmC1 and TaMYB-D1. The solid frame represents the MYB protein-binding domain, and the dotted frame represents the regulation domain

All of these known genes regulate anthocyanin or flavonol biosynthesis. *AtMYB12* regulates caffeoylquinic acid and flavonol synthesis in tobacco and tomato (Luo et al. 2008) and also induces anthocyanin biosynthesis in wheat coleoptiles (Gao et al. 2011). *AtTT2* is the key gene for the biosynthesis of proanthocyanidins in the testae of *Arabidopsis*. *TaMYB10-A*, *TaMYB10-B*, and *TaMYB10-D* are related to red grain colour in wheat (Himi and Noda 2005). *ZmC1* was the first *MYB* gene regulating anthocyanin biosynthesis to be isolated from maize. Interestingly, *TaMYB-D1* possesses only one intron in its gene structure, while the other *MYB* genes have two introns each, except *TaMYB3* (Fig. 2B). The loss of one intron may cause the exon segment loss and shorter amino acid sequence observed in *TaMYB-D1*. Both *TaMYB3* and *TaMYB-D1* were present in the wheat genome and were located on the homoeologous group 4 and 7 chromosomes, respectively. *TaMYB-D1* was also very close to *TaMYB3* in the phylogenetic tree. The coexistence of

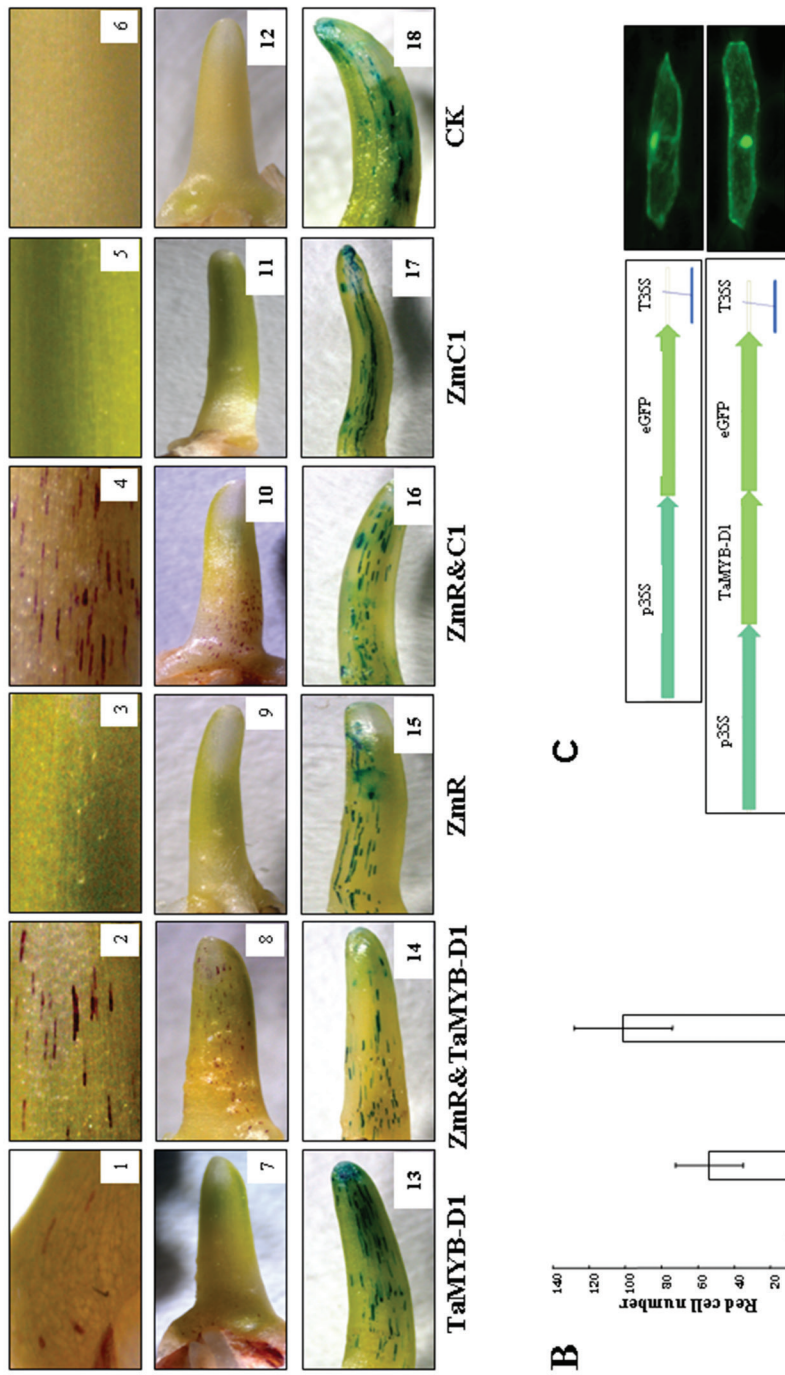

Figure 3. Transient expression experiments in wheat coleoptiles and onion epidermis. A, coleoptiles bombarded with six kinds of plasmids components. 1–6, Enlarged photographs of the coleoptiles after bombardment. 7–12, the coleoptiles after bombardment. 13–18, the coleoptiles with GUS dye after bombardment. B, red cell numbers in the coleoptiles with the bombardment treatment. CK indicates transformation with the empty vector pBRACT214, and R, C1, and TaMYB-D1 represent transformation with pBRACT214-ZmR, pBRACT214-ZmC1, and Pbract214-TaMYB-D1, respectively. pBRACT214-GUS was used in all treatment as a positive bombardment control. C, subcellular location of TaMYB-D1 protein in onion epidermis

TaMYB-D1 and *TaMYB3* may indicate a gene replication event in the wheat genome. Because *ZmC1* has been proven to possess special DNA binding and transcription domains for promoting the expression of the structural genes of anthocyanin biosynthesis (Grotewold et al. 2000), the amino acid sequence of TaMYB-D1 was compared with that of ZmC1. *TaMYB-D1* encoded a protein with 252 amino acids, 21 amino acids shorter than ZmC1. TaMYB-D1 contains an integrated DNA binding domain of 102 amino acids similar to that of ZmC1, with only 12 single-amino-acid substitutions, and a transcription domain of 42 amino acids with a 3-amino-acid deletion and 10 single-amino-acid substitutions compared with that of ZmC1 (Fig. 2C).

Functional verification with transient expression

TaMYB-D1 was very similar to *ZmC1* in the evolutionary analysis. In previous research, *ZmC1* has been shown to induce anthocyanin biosynthesis with the coexpression of the *bHLH* gene *ZmR* (Ahmed et al. 2003; Himi and Noda 2005). In the present experiment, the *bHLH* regulators *ZmR* and *ZmC1* were isolated from maize to compare the function of *TaMYB-D1* with that of *ZmC1*. The coding sequences of *ZmR, ZmC1*, and *TaMYB-D1* were placed after the ubiquitin promoter in the pBRACT214 vector. The transient expressions of *ZmC1* and *TaMYB-D1* induced anthocyanin biosynthesis in the coleoptile cells of 'Opata' in the light treatment with the assistance of *ZmR* (Fig. 3A). The average red cell numbers in each coleoptile for *TaMYB-D1* and *ZmC1* expression in cooperation with *ZmR* were 54 and 101, respectively (Fig. 3B). *ZmC1* thus had nearly double the function of *TaMYB-D1*. Both *ZmC1* and *ZmR* regulated anthocyanin biosynthesis in maize. *ZmC1* may cooperate better with *ZmR* than does *TaMYB-D1* because of coevolution. Only *ZmC1* or *ZmR* yielded no red cells in the pericarps. However, *TaMYB-D1* induced anthocyanin production in some cells without the aid of *ZmR* (Fig. 3A); this result may also be due to coevolution. *TaMYB-D1* exhibited better performance than did *ZmC1* in cooperation with the *bHLH* gene in wheat. The red cell number of each coleoptile was 8 when only *TaMYB-D1* was transformed, much smaller than that observed in conjunction with *ZmR* transformation (Fig. 3B). This result was likely due to the significantly lower expression of the inner *bHLH* gene than of *ZmR* when bombardment was employed for transient expression.

Subcellular locations

The constructs for transient expression in the protoplast were developed as shown in Fig. 3C. The eGFP was ubiquitous in the onion cells transformed with pk7FWG2-R, while the fusion protein of *TaMYB-D1* and eGFP was only present in the nucleus of the epidermal cell (Fig. 3C). It may thus be inferred that *TaMYB-D1* was located in the nucleus, supporting its presumed role as a transcription factor.

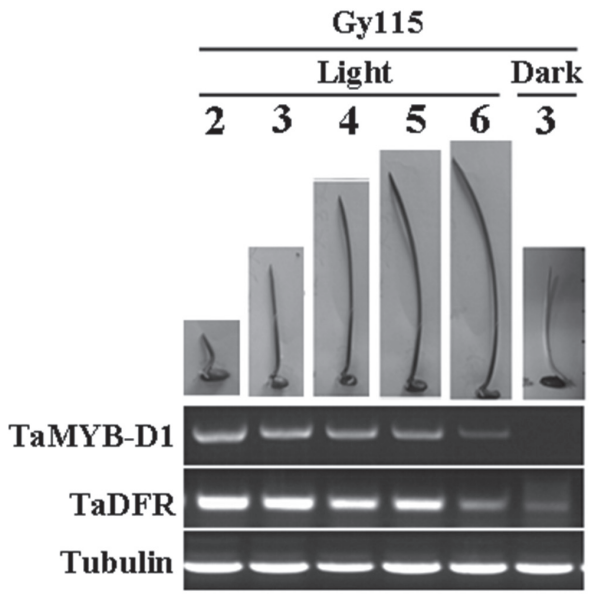

Figure 4. Expression profiles of *TaMYB-D1* over 'Gy115' coleoptile development. "Light" represents coleoptiles grown under light, and "dark" represents coleoptiles grown in dark. The numbers represent days after germination

Expression profiling of TaMYB-D1 *in coleoptiles*

At 2 days after germination, the coleoptiles of 'Gy115' became red in the light treatment. In the dark treatment, anthocyanin was not always seen (Fig. 4). Light should be a necessary environmental factor for anthocyanin biosynthesis in coleoptiles. *TaMYB-D1* was expressed most strongly on the second day of the experiment and declined over the next 5 days of the light treatment. Expression of this gene was not detected in the dark treatment (Fig. 4). Therefore, the expression of *TaMYB-D1* was upregulated by light. The pattern of *TaMYB-D1* expression was very similar to that of anthocyanin content in the coleoptile. The structural gene *TaDFR* was chosen as a representative structural gene in the anthocyanin biosynthesis pathway, and its expression closely followed that of *TaMYB-D1*. Light may have induced *TaMYB-D1* expression in the coleoptiles of 'Gy115', which in turn upregulated the expression of the structural genes that catalyzed anthocyanin biosynthesis.

Discussion

TaMYB-D1 was divided into the same branches as *ZmC1* and other *MYB* proteins that regulate anthocyanin and proanthocyanidin in the phylogenetic analysis. Moreover, *TaMYB-D1* had highly conserved DNA binding and regulating domain sequences similar to

those of *ZmC1*, which regulates anthocyanin biosynthesis in maize. The above results suggest that *TaMYB-D1* is derived from the same ancestor as that of the other *MYB* genes that regulate anthocyanin and has a similar function. Transient expression analysis showed that the *TaMYB-D1* protein was located in the nuclei of plant cells, as is necessary for a regulation factor. Most importantly, the transient expression of *TaMYB-D1* was found to induce anthocyanin biosynthesis in several 'Opata' pericarp cells. In conjunction with the *bHLH* transcription factor *ZmR,* many cells were induced to become red. This result further confirms the activity of *TaMYB-D1* as an *MYB* transcription factor regulating anthocyanin synthesis.

Previous research has shown that the three dominant major genes for the red coleoptile trait should be the MYB regulator genes residing at the short arm of the homoeologous group 7 chromosomes (Ahmed et al. 2006; Himi et al. 2005; Khlestkina et al. 2002). *TaMYB-D1* is a *MYB* regulator gene residing exactly on the short arms of the homoeologous group 7 chromosomes. Moreover, when only *TaMYB-D1* was expressed in the coleoptile cells of 'Opata', some of these cells produced anthocyanin. Therefore, *TaMYB-D1* could recover the red coleoptile phenotype in 'Opata'. Three homoelogous genes, *TaMYB-A1*, *TaMYB-B1*, and *TaMYB-D1*, were isolated from 'Gy115', which possesses red coleoptiles, while only *TaMYB-A1* was found in 'Opata' and 'CS', which have uncoloured coleoptiles. Only one copy of *TaMYB-D1* was expressed in the coleoptiles of 'Gy115', while none were expressed in those of 'Opata' and 'CS'. These observations explain the difference in coleoptile colours between the cultivars and agree with previous classical genetics results showing that one dominant gene decided coleoptile colour in recombinant inbred lines of 'Gy115' and 'Opata' (Liu 2009). Together, these results suggest that *TaMYB-D1* is the key gene controlling red coleoptile colour in wheat.

Acknowledgements

This research was financially supported by the National Natural Science Foundation of China (no. 31071417, 31260322), the West Light Foundation of the Chinese Academy of Sciences, and Pilot Projects of Designer Breeding by Molecular Modules, Chinese Academy of Sciences.

References

Ahmed, N., Maekawa, M., Utsugi, S., Himi, E., Ablet, H., Rikiishi, K., Noda, K. 2003. Transient expression of anthocyanin in developing wheat coleoptile by maize C1 and beta-peru regulatory genes for anthocyanin synthesis. Breeding Sci. **53**:29–34.

Ahmed, N., Maekawa, M., Utsugi, S., Rikiishia, K., Ahmad, A., Noda, K. 2006. The wheat Rc gene for red coleoptile colour codes for a transcriptional activator of late anthocyanin biosynthesis genes. J. Cereal Sci. 44:54–58.

Ban, Y., Honda, C., Hatsuyama, Y., Igarashi, M., Bessho, H., Moriguchi, T. 2007. Isolation and functional analysis of a MYB transcription factor gene that is a key regulator for the development of red colouration in apple skin. Plant Cell Physiol. **48**:958–970.

Borevitz, J.O., Xia, Y., Blount, J., Dixon, R.A., Lamb, C. 2000. Activation tagging identifies a conserved MYB regulator of phenylpropanoid biosynthesis. Plant Cell **12**:2383–2394.

Gao, X., Zhang, L., Zhou, S., Wang, C., Deng, X., Zhang, H., Yang, G., Javeed, H., He, G. 2011. AtMYB12 gene: a novel visible marker for wheat transformation. Mol. Biol. Report **38**:183–190.

Grotewold, E., Sainz, M.B., Tagliani, L., Hernandez, J.M., Bowen, B., Chandler, V.L. 2000. Identification of the residues in the Myb domain of maize C1 that specify the interaction with the bHLH cofactor R. Proc. Nat. Acad. Sci. USA **97**:13579–13584.

Guo, A.Y., Zhu, Q.H., Chen, X., Luo, J.C. 2007. GSDS: a gene structure display server. Yi Chuan **29**:1023–1026. (in Chinese)

Himi, E., Maekawa, M., Noda, K. 2011. Differential expression of three flavanone 3-hydroxylase genes in grains and coleoptiles of wheat. Int. J. Plant Genomics **201**1:369460.

Himi, E., Nisar, A., Noda, K. 2005. Colour genes (R and Rc) for grain and coleoptile upregulate flavonoid biosynthesis genes in wheat. Genome **48**:747–754.

Himi, E., Noda, K. 2004. Isolation and location of three homoeologous dihydroflavonol-4-reductase (DFR) genes of wheat and their tissue-dependent expression. J. Exp. Bot. **55**:365–375.

Himi, E., Noda, K. 2005. Red grain colour gene (R) of wheat is a Myb-type transcription factor. Euphytica **143**:239–242.

Huang, W.J., Sun, W., Lv, H.Y., Luo, M., Zeng, S.H., Pattanaik, S., Yuan, L., Wang, Y. 2013. R2R3-MYB Transcription factor from Epimedium sagittatum regulates the flavonoid biosynthetic pathway. PLoS One 8(8):e70778. doi: 10.1371/journal.pone.0070778.

International Wheat Genome Sequencing Consortium 2014. A chromosome-based draft sequence of the hexaploid bread wheat (*Triticum aestivum*) genome. Science **345**:286–299.

Jia, J.Z., Zhao, S.C., Kong, X.Y., Li, Y.R., Zhao, G.Y., He, W.M., Appels, R., Pfeifer, M., Tao, Y., Zhang, X.Y., Jing, R.L., Zhang, C., Ma, Y.Z., Gao, L.F., Gao, C., Spannagl, M., Mayer, K.F.X., Li, D., Pan, S.K., Zheng, F.Y., Hu, Q., Xia, X.C., Li, J.W., Liang, Q.S., Chen, J., Wicker, T., Gou, C.Y., Kuang, H.H., He, G.Y., Luo, Y.D., Keller, B., Xia, Q.J., Lu, P., Wang, J.Y., Zou, H.F., Zhang, R.Z., Xu, J.Y., Gao, J.L., Middleton, C., Quan, Z.W., Liu, G.M., Wang, J., Yang, H.M., Liu, X., He, Z.H., Mao, L., Wang, J., Consor, I.W.G.S. 2013. *Aegilops tauschii* draft genome sequence reveals a gene repertoire for wheat adaptation. Nature **496**:91–95.

Khlestkina, E.K., Pestsova, E.G., Röder, M.S., Börner, A. 2002. Molecular mapping, phenotypic expression and geographical distribution of genes determining anthocyanin pigmentation of coleoptiles in wheat (*Triticum aestivum* L.). Theor. Appl. Genet. **104**:632–637.

Khlestkina, E.K., Röder, M.S., Salina, E.A. 2008. Relationship between homoeologous regulatory and structural genes in allopolyploid genome – a case study in bread wheat. BMC Plant Biology **8**:88.

Kobayashi, S., Yamamoto, N.G., Hirochika, H. 2005. Association of VvmybA1 gene expression with anthocyanin production in grape (*Vitis vinifera*) skin – colour mutants. J. Japanese Soc. for Horticultural Sci. **74**:196–203.

Li, W.L., Faris, J.D., Chittoor, J.M., Leach, J.E., Hulbert, S.H., Liu, D.J., Chen, P.D., Gill, B.S. 1999. Genomic mapping of defense response genes in wheat. Theor. Appl. Genet. **98**:226–233.

Ling, H.Q., Zhao, S.C., Liu, D.C., Wang, J.Y., Sun, H., Zhang, C., Fan, H.J., Li, D., Dong, L.L., Tao, Y., Gao, C., Wu, H.L., Li, Y.W., Cui, Y., Guo, X.S., Zheng, S.S., Wang, B., Yu, K., Liang, Q.S., Yang, W.L., Lou, X.Y., Chen, J., Feng, M.J., Jian, J.B., Zhang, X.F., Luo, G.B., Jiang, Y., Liu, J.J., Wang, Z.B., Sha, Y.H., Zhang, B.R., Wu, H.J., Tang, D.Z., Shen, Q.H., Xue, P.Y., Zou, S.H., Wang, X.J., Liu, X., Wang, F.M., Yang, Y.P., An, X.L., Dong, Z.Y., Zhang, K.P., Zhang, X.Q., Luo, M.C., Dvorak, J., Tong, Y.P., Wang, J., Yang, H.M., Li, Z.S., Wang, D.W., Zhang, A.M., Wang, J. 2013. Draft genome of the wheat A-genome progenitor *Triticum urartu*. Nature **496**: 87–90.

Liu, B.L. 2009. Genetic and Molecular Analysis of Seed Anthocyanin Accumulation in the Purple Wheat Variety Gaoyuan 115. University of Chinese Academy of Sciences. Beijing, China. (in Chinese)

Luo, J., Butelli, E., Hill, L., Parr, A., Niggeweg, R., Bailey, P., Weisshaar, B., Martin, C. 2008. AtMYB12 regulates caffeoyl quinic acid and flavonol synthesis in tomato: expression in fruit results in very high levels of both types of polyphenol. The Plant J. **56**:316–326.

Mano, H., Ogasawara, F., Sato, K., Higo, H., Minobe, Y. 2007. Isolation of a regulatory gene of anthocyanin biosynthesis in tuberous roots of purple-fleshed sweet potato. Plant Physiol. **143**:1252–1268.

McClintock, B. 1950. The origin and behavior of mutable loci in maize. Proc. Nat. Acad. Sci. USA **36**:344–355.

Peel, G.J., Pang, Y.Z., Modolo, L.V., Dixon, R.A. 2009. The LAP1 MYB transcription factor orchestrates anthocyanidin biosynthesis and glycosylation in Medicago. Plant J. **59**:136–149.

Porret, N., Cousins, P., Owens, C. 2006. DNA sequence variation within the promoter of VvmybA1 associates with flesh pigmentation of intensely coloured grape varieties. Hortscience **41**:1049–1049.

Quattrocchio, F., Wing, J., van der Woude, K., Souer, E., de Vetten, N., Mol, J., Koes, R. 1999. Molecular analysis of the anthocyanin2 gene of petunia and its role in the evolution of flower colour. Plant Cell **11**:1433–1444.

Shoeva, O.Y., Gordeeva, E.I., Khlestkina, E.K. 2014. The regulation of anthocyanin synthesis in the wheat pericarp. Molecules **19**:20266–20279.

Takos, A.M., Jaffe, F.W., Jacob, S.R., Bogs, J., Robinson, S.P., Walker, A.R. 2006. Light-induced expression of a MYB gene regulates anthocyanin biosynthesis in red apples. Plant Physiol. **142**:1216–1232.

Tamura, K., Dudley, J., Nei, M., Kumar, S. 2007. MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol. Biol. and Evol. **24**:1596–1599.

This, P., Lacombe, T., Cadle-Davidson, M., Owens, C.L. 2007. Wine grape (*Vitis vinifera* L.) colour associates with allelic variation in the domestication gene VvmybA1. Theor. Appl. Genet. **114**:723–730.

von Arnim, A. 2007. Subcellular localization of GUS- and GFP-Tagged proteins in onion epidermal cells. CSH Protoc 2007: http://cshprotocols.cshlp.org/content/2007/2/pdb.prot4689.long.

Winkel-Shirley, B. 2001. Flavonoid biosynthesis. A colourful model for genetics, biochemistry, cell biology, and biotechnology. Plant Physiol. **126**:485–493.

Yan, Z., Wan, Y., Liu, K., Zheng, Y., Wang, D. 2002. Identification of a novel HMW glutenin subunit and comparison of its amino acid sequence with those of homologous subunits. Chinese Sci. Bulletin **47**:220–225.