TaMYB3, Encoding a Functional MYB Transcriptor, Isolated from the Purple Pericarp of *Triticum aestivum*

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Purple pericarp is an interesting and useful trait in *Triticum aestivum,* but the molecular mechanism behind this phenotype remains unclear. The allelic variation in the MYB transcriptors is associated with the phenotype of pigmented organs in many plants. In this study, a *MYB* transcription factor gene, *TaMYB3*, was isolated using homology-based cloning and a differentially expressed gene mining approach, to verify the function of the MYB transcriptor in the purple pericarp. The coding sequence of *TaMYB3* in cultivar Gy115 was the same as that in cultivar Opata. *TaMYB3* was localized to FL0.62–0.95 on chromosome 4BL. The TaMYB3 protein contains DNA-binding and transcription-activation domains, and clustered on a phylogenetic tree with the MYB proteins that regulates anthocyanin and proanthocyanin biosynthesis. TaMYB3 localized in the nuclei of *Arabidopsis thaliana* and wheat protoplasts after it was transiently expressed with PEG transformation. TaMYB3 induced anthocyanin synthesis in the pericarp cells of Opata in the dark in collaboration with the basic helix–loop–helix protein ZmR, which is also the function of ZmC1. However, TaMYB3 alone did not induce anthocyanin biosynthesis in the pericarp cells of the white grain wheat cultivar Opata in the light after bombardment, whereas the single protein ZmR did. Light increased the expression of *TaMYB3* in the pericarp of Gy115 and Opata, but only induced anthocyanin biosynthesis in the grains of Gy115. Our results extend our understanding of the molecular mechanism of the purple pericarp trait in *T. aestivum.*

Keywords: MYB transcriptor, purple pericarp, anthocyanin biosynthesis, *Triticum aestivum*

Introduction

Anthocyanin is a water-soluble pigment that occurs ubiquitously in terrestrial plants, except cacti and beets. This class of compound endows plant organs with a red, pink, blue, or purple color. Anthocyanin also has health-giving properties, including antioxidant, antiviral, and antitumor activities (Baublis et al. 2000). Because it is rich in anthocyanins, purple-grained wheat is increasingly regarded as a health food.

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Purple-grained wheat can also be used as an effective marker of the natural outcrossing rate (Griffin 1987). The purple pericarp trait in common wheat was once believed to have originated from the tetraploid wheat of Ethiopia, and is dominant over white grain and red grain (Zeven 1991). Two complementary genes, *Pp1* and *Pp3*, located on chromosomes 7B and 2A, respectively, control the purple grain trait (Khlestkina et al. 2010). There are also genes homologous to *Pp1* on chromosomes 7A and 7D (Tereshchenko et al. 2012; Gordeeva et al. 2015). The purple grain trait in common wheat has been widely studied with classical genetics, but its molecular mechanism has not been fully explained, because the genome of common wheat is large, with three homologous chromosomes.

The key genes in the anthocyanin biosynthesis pathway have been isolated by mutant analyses and map-based cloning in *Arabidopsis*, maize, tobacco, and other model plants with small genomes, because the mutants are readily observable. The main structural genes of anthocyanin biosynthesis include phenylalanine ammonia lyase, chalcone synthase, chalcone isomerase, flavanone 3-hydroxylase, flavonoid 3-hydroxylase, dihydro-flavonol 4-reductase, leucoanthocyanidin dioxygenase, and flavonoid 3-O-glucosyltrans-ferase (Dunlop and Curtis 1991). The metabolism and regulation of anthocyanin biosynthesis are mainly achieved through the regulated expression of structural genes (Gion et al. 2012; Xu et al. 2015), and three types of transcription factors, MYB, bHLH, and WD40, regulate the expression of these structural genes (Broun 2005; Koes et al. 2005). The allelic variation in the *MYB* genes plays a key role in the color differentiation of natural plant populations. For instance, nonfunctional *VvMYBA1* is present in the white grape cultivar Pinot Noir, and the restoration of *VvMYBA1* causes the red skin of the grape cultivar Pinot Blanc as a result of intra-long-terminal-repeat (intra-LTR) recombination within a retrotransposon in the gene's promoter (Kobayashi et al. 2005). Similar functional MYB regulators also exist in *Arabidopsis* (*AtMYB75* [*PAP1*]) and *AtMYB90* [*PAP2*]) (Borevitz et al. 2000), petunia (*AN2*) (Quattrocchio et al. 1999), sweet potato (*MYB1*) (Mano et al. 2007), legumes (*LAP1*) (Peel et al. 2009), and *Epimedium sagitta-tum* (*EsMYBA1*) (Huang et al. 2013). Among the genes associated with anthocyanin bio-synthesis in *Triticum aestivum*, the red grain (*R*) and red coleoptile (*Rc*) genes have been shown to encode R2R3-MYB transcription factors that regulate proanthocyanidin and anthocyanin biosynthesis in grains and coleoptiles, respectively (Himi et al. 2011; Himi and Taketa 2015; Wang et al. 2016). The candidate gene for the purple pericarp trait in common wheat, *Pp3*, is the basic helix–loop–helix (bHLH) transcriptor *TaMYC1* (Shoeva et al. 2014; Liu et al. 2016), and the *Pp-1* genes are thought to be orthologues of maize *C1* and rice *OsC1*, which encode MYB-like transcription factors responsible for the activation of the structural genes encoding various enzymes that participate in anthocyanin synthesis (Saitoh et al. 2004; Khlestkina 2013). However, the function of the MYB tran-scriptor in the purple grain trait of common wheat has not been analyzed.

In this study, a MYB transcription factor gene, *TaMYB3*, was isolated from purple grains with a homology cloning technology based on *ZmC1* and *OsC1*. The chromo-somal site, subcellular localization, functional verification, and expression profile of the gene were analyzed to evaluate the role of *TaMYB3* in the anthocyanin biosynthesis un-derlying the purple grain trait.

Materials and Methods

Plant materials

Wheat cultivar Gy115, with the purple grain trait, was bred at the Northwest Institute of Plateau Biology, Chinese Academy of Sciences. The white grain wheat cultivars Opata, *Aegilops tauschii* AS69 (Accession number: PI603252), and *T. urartu* IE29-1 (Accession number: PI428183), the chromosomal substitution lines of Chinese Spring (CS) in durum wheat Langdon (Joppa and Williams 1988) and the chromosomal deletion lines of CS (Endo and Gill 1996) were provided by the National Plant Germplasm System (NPGS) of the United States Department of Agriculture (http://www.ars-grin.gov/).

DNA and RNA extraction

All samples were collected in liquid nitrogen, and stored in −80 °C before DNA and RNA extraction. DNA was extracted using the CTAB method, and total RNA was extracted from the samples with TRIzol Reagent (Invitrogen, Burlington, ON, Canada), according to the manufacturer's protocol. The DNA and RNA contents were measured with the Bio-Photometer Plus (Eppendorf), and 2 µg of RNA was used for the reverse transcription reactions.

PCR and semi-quantitative PCR

PCR amplification was performed with Phusion High-Fidelity DNA Polymerase (Thermo-Fisher Scientific, Shanghai, China) in the GeneAmp PCR System 9700 (Thermo-Fisher Scientific), with the following thermal cycling parameters: 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; followed by a final extension of 5 min at 72 °C. The PCR products were extracted from 1.0% agarose gels using the Tiangen TIANgel Midi Purification Kit (Tiangen) and cloned into the pGEM-T Easy vector (Promega Corporation, Madison, Wisconsin, USA). *Escherichia coli* DH5α cells were then transformed with the recombinant plasmid, and six positive clones were sent to a commercial company (Huada Gene, Shenzheng, China) for sequencing. The nucleotide sequence of *TaMYB3* was deposited in GenBank under accession number KX373488. All the primers used in this study are listed in Table S1*. In all the semi-quantitative RT–PCR experiments conducted in this study, the amplification of the wheat tubulin gene transcripts was used to normalize the cDNA contents of the various reverse transcription mixtures. Thirty-two thermocycles were used to check the linearity of the amplifications. The reproducibility of the transcription patterns determined with semi-quantitative PCR was tested in at least three independent assays.

*Further details about the Electronic Supplementary Material (ESM) can be found at the end of the article.

Bioinformatic analysis

The sequences were aligned with the Vector NTI 10 software (Thermo-Fisher Scientific). Primers were designed with the Primer5 software (Premier Biosoft, Palo Alto, CA, USA). Phylogenetic trees were constructed with the MEGA 4 software (Tamura et al. 2007), and the protein domains were predicted with the web software SMART (http://smart.em BL-heidelberg.de/).

Transient expression in protoplasts transformed with the polyethylene glycol (PEG) method

For the subcellular localization experiment, the coding sequence of *TaMYB3* without the termination codon was ligated into the PMNG vector after the ubiquitin promoter and before the enhanced green fluorescent protein gene (*eGFP*). Protoplasts were prepared according to Sheen (2002) and Yoo et al. (2007). The leaves of *Arabidopsis* (without blossom) were used to prepare the protoplasts, and etiolated wheat seedlings 7 days after germination were used to prepare the wheat protoplasts. The protoplasts were transformed with plasmid PMNG:TaMYB3 or PMNG (the positive control) using a PEG method, based on the method of Sheen (2002).

Expression of TaMYB3 *in grains after dark or light treatment*

To study the reaction of *TaMYB3* and anthocyanin biosynthesis to light, two rows of spike glumes were removed from Gy115 14 days after blossoming as the light treatment, and the remaining two rows were retained as the dark treatment. During the daytime, the spike glumes can reduce the light intensity, so grains without glumes must be exposed to direct sunlight. The grains subjected to the dark and light treatments were stored at −80 °C before photography and the gene expression analysis. The photographs were taken with an Canon D500 SLR camera.

Transient expression in pericarps

The coding sequences of the *TaMYB3*, *ZmC1*, and *ZmR* genes from maize were cut with restriction enzymes *BamHI* and *KpnI* and ligated into the PUBI vector after the *Ubi* promoter. To transform the cells, the plasmids were shot into them with a gene gun, according to the method of Ahmed, for functional verification (Ahmed et al. 2003). The grains were selected for bombardment 14 days after blossoming. After bombardment, the Petri dishes containing the grains of Gy115 were wrapped in aluminum for the dark treatment, and the Petri dishes of Opata were placed directly in a light incubator for the light treatment (light intensity 100 μmol/s/m², 23 °C, 16/8 h light/dark). The grains were observed and photographed after 2 days under a stereomicroscope (Leica Co.).

Results

Molecular characteristics of TaMYB3

After the sequences of *ZmC1* of maize, *AtTT2* of *A. thaliana*, and *OsC1* of rice were aligned, the conserved primers TaC1-F and TaC1-R spanning two introns were designed to isolate the *MYB* genes regulating anthocyanin biosynthesis in pigmented grains using homology-based cloning (Fig. S1A). Previous studies have demonstrated that anthocyanin biosynthesis is induced by light in the purple pericarp tissue of wheat (Wang et al. 2011). The grains of Gy115 treated with light or dark were used as the substrates in which to distinguish the differential expression of the *MYB* gene. A cDNA amplification product was isolated from 7-day grain from wheat cultivar Gy115 treated with light for 2 days, but was not isolated from grains exposed to the dark treatment (Fig. S1B). The nucleotide sequence of amplified fragment was very similar to that of the *MYB* genes in the alignment. Contigs TA59663_4565 and TA59683_4565 were recovered from the expressed sequence tag (EST) database for wheat at the TIGR website with a BLAST search. The two contigs were assembled into one sequence of 1231 bp. *TaMYB3* was isolated from Gy115 and Opata based on the assembled sequence. The genomic sequence of *TaMYB3* in Opata differed by a single base in the intron from that in Gy115, and did not influence the amino acid sequence of the encoded protein.

On a phylogenetic tree, *TaMYB3*, *ZmC1*, *ZmP*, and *AtTT2* clustered on the same branch (Fig. 1B). With the exception of *TaMYB3*, all these genes have been shown to encode proteins that regulate anthocyanin or proanthocyanin biosynthesis. ZmC1 and TaMYB3 share 54% sequence identity, and in previous research, ZmC1 has been shown to have special DNA-binding and transcription-activation domains that promote the expression of the structural genes involved in anthocyanin biosynthesis (Grotewold et al. 2000). Based on these findings, the amino acid sequence of TaMYB3 was compared with that of ZmC1. Although TaMYB3 is 29 amino acids shorter than ZmC1, TaMYB3 has an integrated DNA-binding domain of 101 amino acids, which is similar to that of ZmC1, with only 13 single-amino-acid substitutions, and a transcription domain of 39 amino acids with a 7-amino-acid deletion and 12 single-amino-acid substitutions (Fig. 1A). Interestingly, *TaMYB3* has only one intron, whereas the other *MYB* genes have two introns in their gene structures (Fig. 1C).

Chromosomal position and subcellular localization

IE29-1 (*T. urartu*) and AS69 (*Ae. tauschii*), the Langdon (CS) chromosome substitution lines and the CS chromosome deletion lines were used to identify the position of *TaMYB3* on the wheat chromosomes. *TaMYB3* could not be amplified from the genome of IE29-1 (genome donor A) or AS69 (genome donor D), signifying that *TaMYB3* does not occur in genome A or D (Fig. 2A). However, *TaMYB3* was detected in the Langdon genome, whereas it was not amplified from the substitution line lacking the 4B chromosome of Langdon but containing the 4D chromosome of CS (Fig. 2A). Therefore, *TaMYB3* must occur on chromosome 4B. To confirm the position of *TaMBY3* on chromosome 4B, a se-
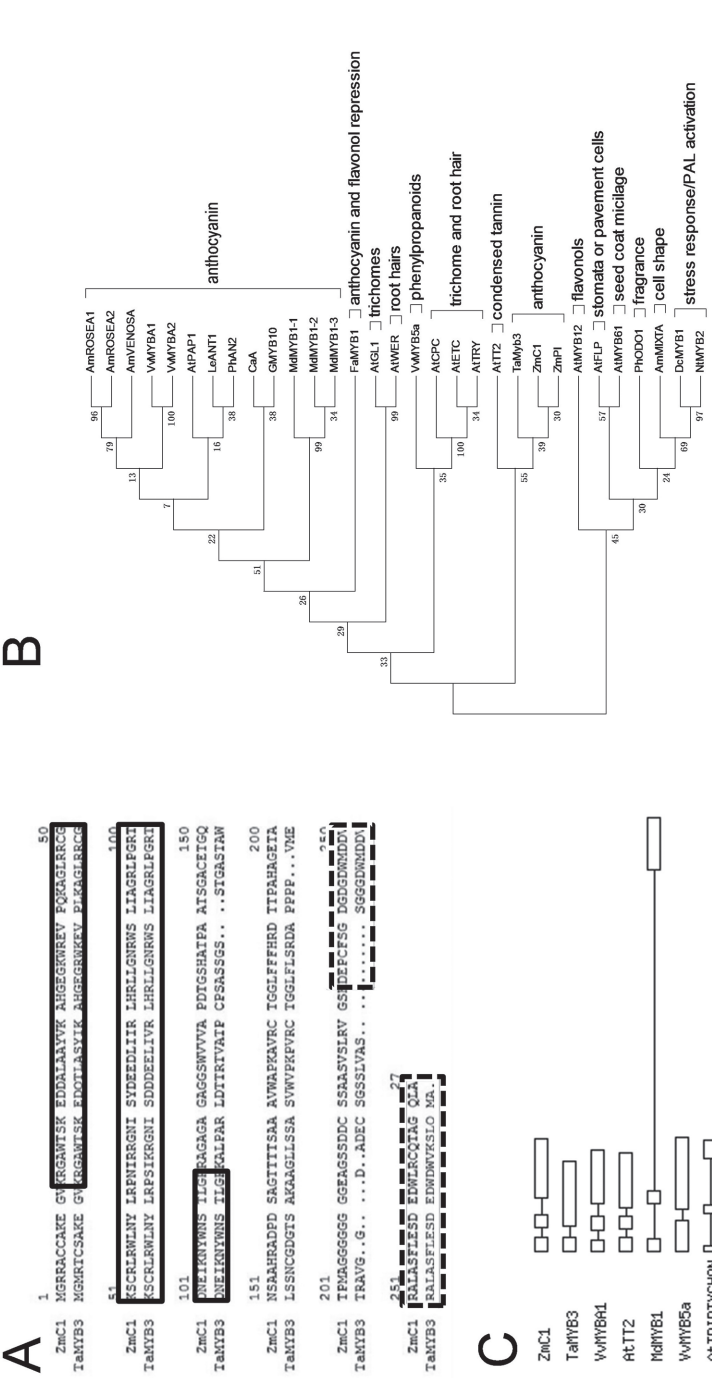
Figure 1. Bioinformation anlysis. A, sequence alignment of ZmC1 and TaMYB3. The bold frame represents the binding domain of the MYB protein, and the dotted frame represents the regulation domain. B, phylogenetic tree of MYB proteins based on MYB domain (AmMIXTA, CAA55725; AmROSEA1, ABB83826; AmROSEA2, 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; ZmPl, AAA19821; TaMYB3, KX373488). C, the structure of partial *MYB* genes (*VvMYBA1*, AB111101; *ZmC1*, AF320613; *MdMYB1*, DQ222406; *AtTT2*, NC_003076; VvMYB5a, NC_0120014.3; AtTRIPTYCHON, NC_003076.8; *TaMYB3*, KX373488). The frames showed the extrons, and the lines showed the introns
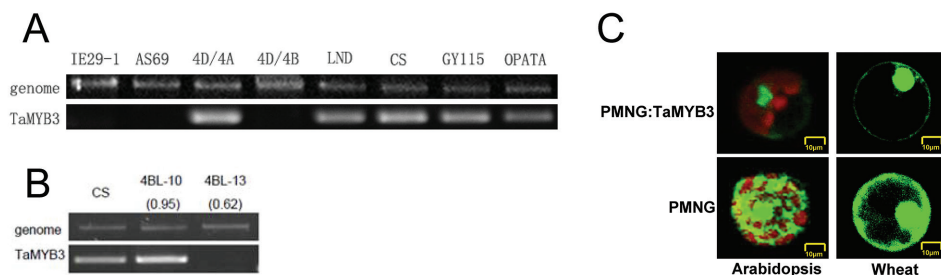
Figure 2. Chromosome residential site and subcellular location of *TaMYB3*. (A) IE29-1, Einkorn wheat (genome A); AS69, *Ae. tauschii* (genome D); 4D/4A, Langdon 4A (CS 4D); 4D/4B, Langdon 4B (CS 4D); LND, Langdon, *Triticum durum* (genome AB); CS, Chinese Spring, common wheat (genome ABD); Gy115, common wheat, Opata, common wheat. Genome land was 100 ng genome DNA, and TaMYB3 land was amplification products with TaMYB3 primer. (B) CS, Chinese Spring. 4BL10, CS with 95% of long arm of chromosome 4B; 4BL13, CS with 62% of long arm of chromosome 4B. Genome land was 100 ng genome DNA, and TaMYB3 land was amplification products with TaMYB3 primer. (C) Protoplasts under confocal laser scanning microscopy after transient expression with plasmids

ries of CS 4B deletion lines was used. *TaMBY3* was detected at 4BL10 (FL0.95), but not at 4BL13 (FL0.62) (Fig. 2B), so *TaMYB3* should occur at FL0.62–0.95 on the long arm of chromosome 4B (Fig. S2). When *Arabidopsis* and wheat protoplasts were transformed with PMNG, eGFP was ubiquitously expressed in the protoplasts, whereas the fusion protein TaMYB3–eGFP was only present in the nuclei of the protoplasts (Fig. 2C). Therefore, we infer that TaMYB3 is located in the nuclei.

Expression profile of TaMYB3 *in grains treated with light or dark*

The light treatment for two days induced anthocyanin biosynthesis in the grains of Gy115, but not in those of Opata. The expression of *TaMYB3* differed from the expression of anthocyanin biosynthesis, and was greater after the light treatment than after the dark treatments in both Gy115 and Opata (Fig. 3). This phenotype may be attributable to the
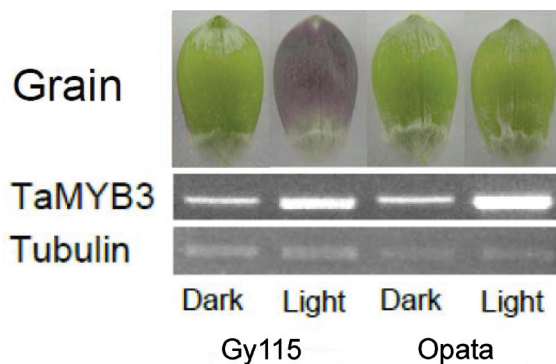
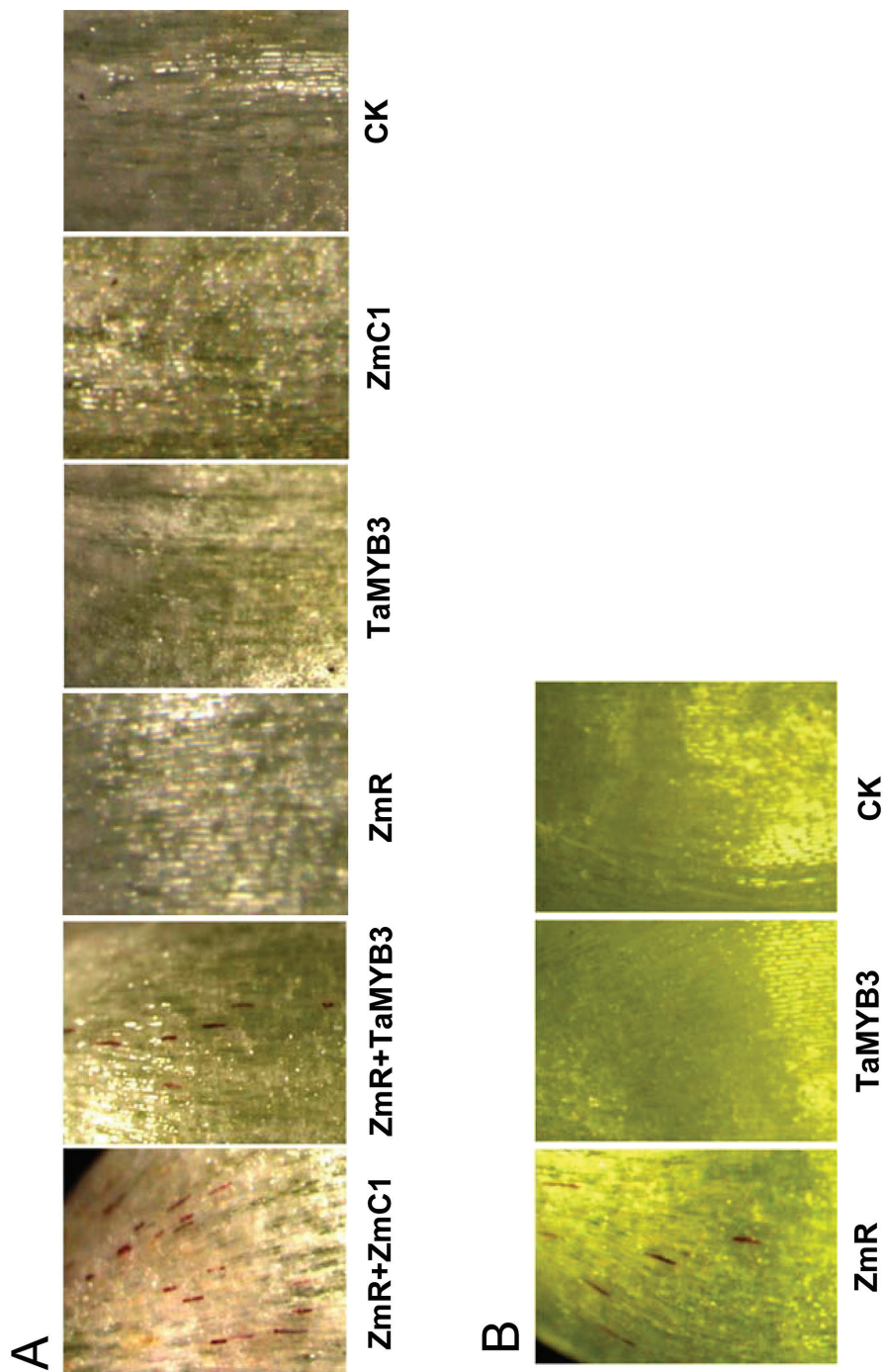*Figure 3.* Relative transcript levels of *TaMYB3* and *Tubulin* in grains with dark treatment and light treatment

Figure 4. Transient expression of *TaMYB3* with bombardment in pericarps of Opata. CK signifies that it was transformed with empty vector PUBI, and ZmR, ZmC1 and TaMYB3, respectively, represent, the treatments with PUBI:ZmR, PUBI:ZmC1, and PUBI:TaMYB3. (A) The gains were checked and photographed after 2 d in the dark treatment. (B) The grains were checked and photographed after 2 d in the light treatment

fact that light induced the expression of *TaMYB3* and anthocyanin biosynthesis in Gy115, and that anthocyanin provided the pericarp with some protection from light. Light induced the expression of *TaMYB3*, but did not cause anthocyanin biosynthesis without the help of other genes related to anthocyanin biosynthesis in the grains of Opata.

Transient expression of *TaMYB3 in the pericarp*

TaMYB3 was very similar to *ZmC1* in the evolutionary analysis, and in previous research, *ZmC1* was shown to induce anthocyanin biosynthesis when coexpressed with the *bHLH* gene (Cone et al. 1986; Ahmed et al. 2003). In the present study, the bHLH regulators *ZmR* and *ZmC1* were isolated from maize to compare the functions of *TaMYB3* and *ZmC1*. The transient expressions of *TaMYB3* or *ZmC1* induced anthocyanin biosynthesis in the pericarp cells of GY115 after dark treatment, with the assistance of *ZmR*. However, the expression of *TaMYB3*, *ZmC1*, or *ZmR* alone did not produce red cells in the pericarps (Fig. 4A). Therefore, *TaMYB3* functions in regulating anthocyanin biosynthesis in a similar to *ZmC1*. TaMYB3, ZmC1, or ZmR was also transiently expressed in the white pericarps of Opata in the light. ZmR alone induced anthocyanin biosynthesis in the pericarp cells of Opata with light treatment, whereas TaMYB3 alone did not produce the same phenotype (Fig. 4B).

Discussion

In this study, we hoped to identify the gene encoding the key factor that activates anthocyanin biosynthesis in the wheat pericarp, as in our recent study (Liu et al. 2016). Compared with the transcriptomic analysis used in the previous study, we used homology-based cloning and differentially expressed gene mining here. The MYB transcriptors ZmC1, AtTT2, and OsC1 have been shown to regulate anthocyanin biosynthesis in maize, *Arabidopsis*, and rice, respectively (Cone et al. 1986; Saitoh et al. 2004; Wei et al. 2007). The *MYB* transcriptor sequences conserved in maize, *A. thaliana*, and rice should also be conserved in common wheat. TaMYB3, the protein derived from the conserved nucleotide sequence detected, has DNA-binding and regulatory domains involved in anthocyanin biosynthesis. On a phylogenetic tree, TaMYB3 clustered on the same branch as ZmC1 and other proteins that regulate anthocyanin and proanthocyanin biosynthesis. The sequence-conservation-based strategy used to isolate the homologous gene was very efficient. The transient expression of *TaMYB3* induced anthocyanin biosynthesis in the pericarp of cultivar Gy115 during dark treatment, with the co-operation of the bHLH transcription factor *ZmR*, which is similar to *ZmC1* (Ahmed et al. 2003). *TaMYB3* is expressed in purple grains and is induced by light. Following the increased expression of *TaMYB3*, anthocyanin began to be synthesized. Therefore, TaMYB3 regulates anthocyanin biosynthesis and could be responsible for the purple pericarp, indicating that TaMYB3 is involved in anthocyanin biosynthesis in the purple pericarp, even if other MYB transcriptors also participate in the process.

According to our experiments, the *TaMYB3* gene occurs at FL0.64–0.95 on wheat chromosome 4BL, unlike *R*, *Rc*, and *Pp*. *R* and *Rc* occur on chromosomes 3 and 7, respectively (Himi et al. 2011; Himi and Taketa 2015; Wang et al. 2016), and *Pp1* and *Pp3*, which control the purple grain trait, are located on chromosomes 7B and 2A, respectively (Khlestkina et al. 2010). Therefore, the chromosomal site of *TaMYB3* is clearly inconsistent with the chromosomal locations of the two complementary genes responsible for the purple grain trait of wheat. The genomic and cDNA sequences of *TaMYB3* in Opata are the same as those in Gy115, except for one single-nucleotide difference in the intron. Its relative expression profile in the grains of Opata is also similar to that in the grains of Gy115. Therefore, *TaMYB3* is not the key gene responsible for the purple pericarp of common wheat. However, its chromosomal location at FL0.64–0.95 is close to another locus on 4BL that determines the purple coleoptile trait (Khlestkina et al. 2002). The transient expression of TaMYB3 alone did not induce anthocyanin biosynthesis in the white pericarp of Opata, whereas the transient expression of ZmR did. As mentioned in the introduction, the bHLH transcriptor TaMYC1 was thought to be encoded by the candidate gene for *Pp3* (Shoeva et al. 2014; Liu et al. 2016). The transient expression of *ZmR* compensated for *TaMYC1* in the white pericarp of Opata. This also suggests that the native Opata TaMYB3 protein interacted with the transiently expressed ZmR, resulting in anthocyanin synthesis under light condition. *Pp3* requires the help of *Pp1* to exercise its function in inducing anthocyanin biosynthesis in the pericarp of common wheat (Khlestkina et al. 2010). More work is required to identify the gene encoding *Pp1*.

In this study, a typical MYB transcriptor gene, *TaMYB3*, was isolated from the purple pericarp of common wheat. TaMYB3 regulates anthocyanin biosynthesis, and is probably involved in the anthocyanin biosynthesis that causes the purple pericarp. However, *TaMYB3* is not the key gene responsible for the purple pericarp because its chromosomal location is inconsistent with this role and there is no obvious difference in its expression in the purple cultivar Gy115 and the white cultivar Opata. However, these results should facilitate the further exploration of the molecular mechanism underlying the purple pericarp trait.

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Electronic Supplementary Material (ESM)

Electronic Supplementary Material (ESM) associated with this article can be found at the website of CRC at http://www.akademiai.com/content/120427/

Electronic Supplementary *Table S1*. Names and sequences of the primers used in this study

Electronic Supplementary *Figure S1*. Conserve primers design and amplification with cDNA of Gy115 grain 7 d after blossom. **A**, TaC1-F and TaC1-R were designed in the first and third extrons close to the introns. The arrows show the sites of the primers. (*OsC1*, KJ934847.1; *AtTT2*, NC_003076; *ZmC1*, AF320613), **B**, 7(L) represents the amplification product from cDNA of Gy115 7 d after blossom with dark treatment (Land 1), and 7(d) is the dark treatment. The objective land was 220 bp (Land 2). Land 3 was the marker

Electronic Supplementary *Figure S2*. The *TaMYB3* location on chromosome 4BL