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Dosage effect of anthocyanin biosynthesis in purple-grained wheat (*Triticum aestivum* L.)

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Abstract Purple color of the wheat grain is due to the accumulation of anthocyanins in the pericarp and is genetically controlled by two complementary regulatory genes, namely *TaPpm1* and *TaPpb1*. To study their complementary mechanism, six crosses were made with 3 pairs of nonpurple-grained DH lines and an EMS-induced mutant, which were derived from the white-grained parent Liangxing99 (LX99) and/or purple-grained Nongda3753 (ND3753), respectively. As expected, the purple character was successfully recovered in all the F_1 progeny, although their seed color was visually lighter than that of ND3753 to various extents. The cross between DH41 (*TaPpm1a*/*TaPpb1b*) and DH33 (*TaPpm1d*/*TaPpb1a*), which showed the deepest seed color in all of the progeny, was used to further investigate related genes expression and anthocyanin accumulation. Measurement of anthocyanin content across five grain developing stages showed that the accumulation of anthocyanin in seeds of DH41/DH33 was lower and later than that of ND3753. Detection of expression patterns of two regulatory genes revealed that *TaPpb1* expressed quite consistently in double-locus heterozygous, double-locus dominant homozygous, and single-locus dominant homozygous, whereas the expression of *TaPpm1* showed a significant difference in different states. It was highly expressed in the double-locus homozygous genotype in the early grain developing stage and increased expression in double-locus heterozygous in the late stage but remained low level in single-locus homozygous in all stages. Furthermore, expression patterns of seven structural genes involved in anthocyanin biosynthesis were investigated, among which *F3'H*, *DFR*, and *ANS* were in accordance with that of *TaPpm1* and the anthocyanin accumulation. Thus, the dosage effect of wheat purple grain color was a result of delayed expression of one of the two regulatory genes, namely *TaPpm1* in the heterozygous genotype, and consequently the reduced expression of middle and later structural genes in anthocyanin biosynthesis pathway in grain development.

Keywords Wheat · Purple pericarp · Anthocyanin biosynthesis · Dosage effect · Gene expression

Chan Bi and Chaoxiong Wei contributed equally to this work.

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Introduction

Anthocyanins, one of the important natural water-soluble pigments that determine the colors of flowers, fruits, seed coats, stems, and leaves, are widely found in plants. In addition to responding to biological and abiotic stresses to scavenge oxygen free radicals (Fan et al. 2016; Nakabayashi et al. 2014; Dong et al. 2020; Li et al. 2020), and protecting themselves from high-density light damage (Li et al. 2018; Hatier et al. 2013), anthocyanins also have nutritional and healthcare functions for humans in terms of anticancer, disease resistance, and anti-oxidation (Peiffer et al. 2016; Isaak et al. 2017; Mrkvicova et al. 2016). Many studies related to the metabolic pathways of anthocyanins and key genes involved in this pathway have been performed. Plant anthocyanidin biosynthesis belongs to the flavonoid biosynthesis pathway and the precursor of anthocyanin biosynthesis is phenylalanine which is catalyzed by a series of enzymes to finally generate anthocyanins. The main synthase enzymes in this pathway include phenylalanine ammonia-lyase (PAL), chalcone synthase (CHS), chalcone isomerase (CHI), flavanone-3-hydroxylase (F3H), flavanone 3′-hydroxylase (F3′H), flavanone 3′5′-hydroxylase (F3′5′H), dihydroflavonol-4-reductase (DFR), anthocyanidin synthase/leucoanthocyanidin dioxygenase (ANS/LDOX), and uridine diphosphate glucose-flavonoid glucosyltransferase (UFGT), etc. (Martin and Gerats 1993; Mol et al. 1998; Himi et al. 2005). The expression of structural genes encoding these enzymes is mainly regulated by transcription factors (TFs). There are three main types of TFs involved in regulating anthocyanin biosynthesis, namely MYB, bHLH, and WD40 proteins. These TFs can be combined with corresponding cis-acting elements in structural gene promoters independently or synergistically to regulate the temporal and spatial expression of structural genes, thereby regulating the accumulation of anthocyanins in plants (Koes et al. 2005; Petroni and Tonelli 2011).

The accumulation of anthocyanins in the pericarp of purple wheat has been reported by the interaction of two complementary dominant genes. Previous research found that *Pp1* and *Pp3* were the genes that determined the purple pericarp in wheat, which were located on chromosomes 7D (*Pp-D1*) and 2A, respectively (Dobrovolskaya et al. 2006; Tereshchenko et al. 2012). *Pp-1* is a homologous gene of *ZmC1* in maize and *OsC1* in rice. It encodes an MYB TF and activates the structural genes involved in anthocyanin biosynthesis (Reddy et al. 1998; Saitoh et al. 2004; Khlestkina 2013). *Pp3* has been shown to be homologous to *Lc*/*R* encoding a bHLH TF in maize and *Pb*/*Ra* in rice (Khlestkina 2013; Ludwig et al. 1989; Hu et al. 1996). The latest studies identified *TaPpm1* (encoding an MYB TF) as the candidate gene for *Pp1*, and *TaPpb1/TaMYC1* (encoding a bHLH TF) for *Pp3*. There are four variants of *TaPpm1*(*TaPpm1a/b/c/d*) in the genic region, and two variants of *TaPpb1*(*TaPpb1a/b*) in the promoter region (Jiang et al. 2018). Co-expression of *TaMYC1* with *ZmC1* could induce the accumulation of anthocyanins in the coleoptile of white pericarp grains (Zong et al. 2017). The interaction of *TaPpm1* and *TaPpb1* could also co-regulate anthocyanin biosynthesis in the wheat pericarp (Jiang et al. 2018). However, the interaction of these two TFs was most performed in vitro, there was no reported study about the successful generation of progenies with purple grains by crossing with complementary alleles of *TaPpm1* and *TaPpb1*.

Homozygous and heterozygous genotypes have different dosage effects with different numbers of genes, which affects the corresponding phenotypic changes. For example, the dosage of the *tb1-ref* mutant allele affected maize roots, and homozygous *tb1-ref* mutant plants showed a greater shoot and root biomass than heterozygotes, resulting in the similarity of the modern maize homozygous *tb1-ref* mutants to its ancestor Balsas teosinte (Gaudin et al. 2014). The common wheat *AGL6* gene was involved in the development of the spikelet meristem, and its overexpression increased the spikelet number per spike. Heterozygous mutant progenies (aaBb or Aabb) obtained by crossing the tetraploid Kronos single mutant plants (aaBB and AAbb) were partially fertile, while the homozygous mutant plants (aabb) were completely sterile (Kong et al. 2022). Likewise, whether the expression of *TaPpm1* and *TaPpb1* genes had a dosage effect on anthocyanin biosynthesis in pericarp, and grain color was unknown. Additionally, it has been found that different regulatory genes act on different structural genes (Zimmermann et al. 2004; Grotewold et al. 1994; Mano et al. 2007; Yuan et al. 2009; Quattrocchio et al. 2006). In *Arabidopsis*, R2R3-MYB, bHLH, and WD40 form the MBW complex to activate the expression of late

biosynthesis genes, including *F3'H*, *DFR*, *ANS*, and *UFGT* and ultimately lead to the accumulation of anthocyanins in the plant (Chen and Wang 2019). The MYB-bHLH-WD40 complex activates structural genes including *OsCHS*, *OsCHI*, *OsF3'H*, *OsF3H*, *OsDFR*, and *OsANS*, and thus regulates anthocyanin biosynthesis in rice leaves (Zheng et al. 2019). Nevertheless, it is still unclear which structural genes are regulated by the interaction of *TaPpm1* and *TaPpb1* in the anthocyanin biosynthesis pathway of the wheat pericarp.

In this study, several selected white pericarp lines in a DH population were constructed earlier by crossing purple-grained parent Nongda3753 (ND3753) with white-grained parent Liangxing99 (LX99) and an EMS-induced mutant of ND3753 was used to investigate the effects of *TaPpm1* and *TaPpb1* on anthocyanin biosynthesis. The anthocyanin content, the expression of *TaPpm1* and *TaPpb1* as well as the expression of key structural genes related to anthocyanin biosynthesis were measured and compared at various grain developmental stages. In this way, we can also verify there is a dosage effect in homozygous and heterozygous genotypes about *TaPpm1* and *TaPpb1*, and speculate which structural genes are regulated by the interaction of these two TFs. The purpose of this study was to clarify the regulation mechanism of anthocyanin biosynthesis in the purple pericarp of wheat and to provide the molecular basis for cultivating purple wheat.

Materials and methods

Plant materials and experimental design

A DH population generated by crossing a purple pericarp variety ND3753 with a white pericarp variety LX99 (120 DH lines) was used in this study (Tian et al. 2021). Six white pericarp lines among the DH population with known alleles of *TaPpm1* and *TaPpb1*, and an EMS-induced mutant from ND3753 named M564 with white pericarp were chosen and crossed with each other to generate different allele combinations. Two groups of crosses were made within DH lines (DH41/DH33, DH93/DH10, DH103/DH82) and between DH lines and M564 (DH41/M564, DH93/M564, DH103/M564) (Fig. S1). The developing grains of the progenies from the crossing were collected at 12, 17, 22, 27, and 32 days after pollination (DAP), and were stored in a refrigerator at −80 °C. The grains were taken from the middle part of frozen wheat spikes and used for observation, determination of anthocyanin content, and analysis of related gene expression.

Determination of anthocyanin content

The extraction of total anthocyanins used the colorimetric method of Ficco and Zhu (Ficco et al. 2014; Zhu et al. 2015) with some modifications. The grains were ground into a fine powder. A mixture of methanol acidified with 1 N HCl (85:15; v/v) was added to the samples and then stored at 4 °C for 24 h in dark. After centrifugation at 12,000 rpm for 30 s at 4 °C, the supernatants were stored at −20 °C, and re-extraction of the sediments was done iterative procedure. The mixed supernatants were filtered using 0.45 µm regenerated cellulose syringe filters. The absorbance values at wavelengths of 530 nm and 650 nm were measured. The anthocyanin content was quantified as (A530-0.25 × A650)/fresh weight.

DNA extraction and gene cloning

Previous studies have shown that the purple pericarp of wheat is co-regulated by two regulatory genes *TaPpm1* (7D) and *TaPpb1/TaMYC1* (2A) (Jiang et al. 2018). To identify the genotype, we used the published specific primers to amplify *TaPpm1* and *TaPpb1* in ND3753, LX99, M564, and the white pericarp DH lines respectively. Genomic DNA was extracted from the young leaves by the modified cetyltrimethylammonium bromide (CTAB) method (Devi et al. 2013). Then the extracted DNA was used to amplify the promoter and genic regions of *TaPpm1* and *TaPpb1* with Tks Gflex™ DNA Polymerase (TaKaRa). The specific primers previously reported (Table S1) were used to amplify the sequence (Jiang et al. 2018). The reaction system of PCR amplification comprised 10 µl 2 × Gflex PCR Buffer, 2.0 µl template DNA, 2.0 µl mixture of left and right primers (10 µM), 5.6 µl ddH_2O and 0.4 µl Tks Gflex DNA Polymerase to the final volume of 20 µl. The PCR cycling conditions included 1 min at 95 °C; 34 cycles of 10 s at 98 °C, 15 s at 55–63 °C (according to the different *Tm* of primers), and 1–3 min at 68 °C; the

final extension of 5 min at 68 °C. The PCR product was separated by 1% agarose gel electrophoresis.

RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR)

The total RNA was extracted from grains at different developmental stages using the MiniBEST Plant RNA Extraction Kit (TaKaRa), and the first-strand cDNA was obtained by using the PrimeScript RT reagent Kit with gDNA Eraser (TaKaRa). Quantitative real-time PCR (qRT-PCR) was performed using TB Green Premix Ex Taq II (Tli RNaseH Plus) (TaKaRa). *TaActin* was used as an internal reference gene to measure the expression of related genes in the anthocyanin biosynthesis pathway. The reaction system was run in the Bio-Rad CFX96 Touch Real-Time PCR Detection System. The expression levels were calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001). All experiments were performed with three biological replicates. Previously reported primers were used with slight changes (Jiang et al. 2018) and the sequences were shown in Table S1.

Results

Gene cloning and genotype determination

The grain pericarp color of the DH population derived from the cross of purple pericarp ND3753 with white pericarp LX99 could be visually divided into purple and white groups (Fig. 1a), which contained 29 and 91 lines and fitted the separation ratio of 1:3 ($\chi^2 = 0.044$). These results correspond to the genetic mechanism of two complementary dominant genes. Parents' genotype detection by amplifying and sequencing *TaPpm1* and *TaPpb1* revealed that ND3753, LX99, and M564 carried alleles of *TaPpm1a/TaPpb1a*, *TaPpm1d/TaPpb1b*, and *TaPpm1b/TaPpb1a*, respectively (Fig. 2a). Same strategy was used to screen the white pericarp DH lines and found that there were three homozygous genotypes including *TaPpm1d/TaPpb1a*, *TaPpm1a/TaPpb1b* as well as *TaPpm1d/TaPpb1b*. The results of genotyping of some lines were shown in Fig. 2b.

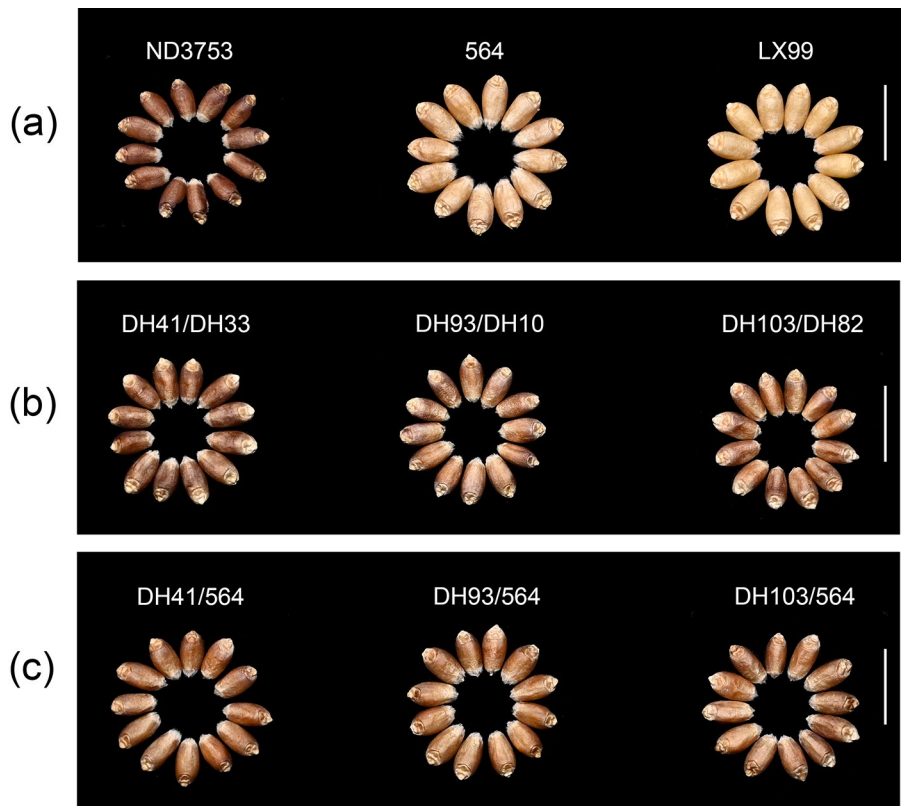

Fig. 1 Identification of wheat pericarp color. **a** Phenotypes of ND3753, M564, and LX99. **b** Phenotypes of the progenies of hybrid combinations by different white pericarp DH lines. **c** Phenotypes of the progenies of hybrid combinations by white pericarp DH lines and M564. Bars, 1 cm

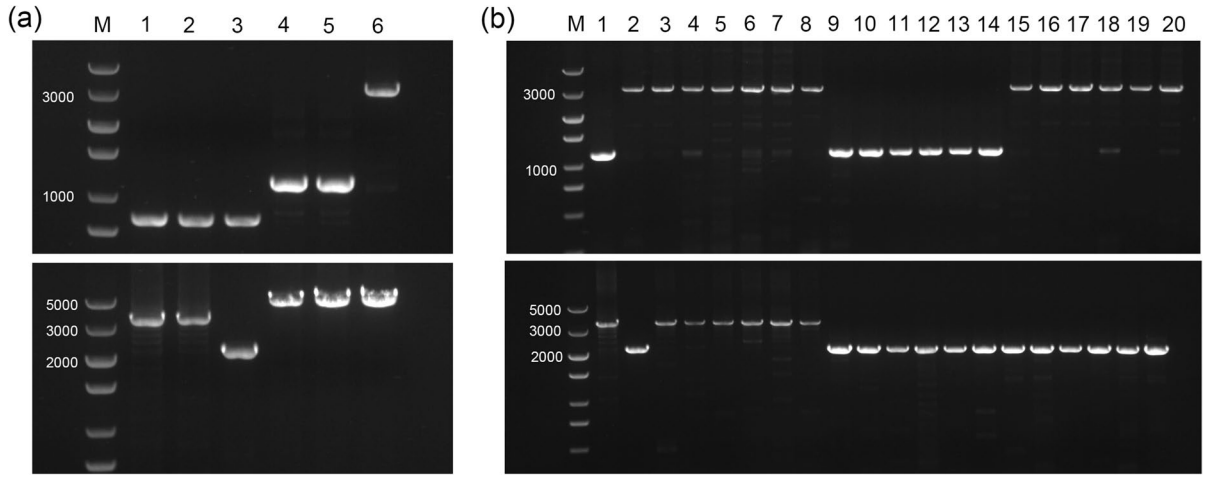

Fig. 2 Cloning of the promoters and genic regions of *TaPpm1* and *TaPpb1* and genotyping of some white pericarp lines in the DH population. **a** Amplification results of promoters and genic regions of *TaPpm1* and *TaPpb1* in ND3753, M564, and LX99. 1, 2, 3, amplification results of promoters in ND3753, M564, and LX99; 4, 5, 6, amplification results of genic regions in ND3753, M564, and LX99. The agarose gel diagram at the top is the amplification results of *TaPpm1*, and at the bottom is the amplification results of *TaPpb1*. **b** Genotyping of *TaPpm1* and *TaPpb1* in some white pericarp lines in the DH population. 1, ND3753; 2, LX99; 3–8, DH lines with *TaPpm1d/TaPpb1a*; 9–14, DH lines with *TaPpm1a/TaPpb1b*; 15–20, DH lines with *TaPpm1d/TaPpb1b*. The agarose gel diagram at the top is the genotyping of *TaPpm1*, and at the bottom is the genotyping of *TaPpb1*

Genetic complementation experiment of two transcription factors

The white pericarp DH lines DH41, DH93, and DH103 with *TaPpm1a/TaPpb1b* as well as DH33, DH10, and DH82 with *TaPpm1d/TaPpb1a* were chosen to cross each other for generating complementary genotype. Meanwhile, DH41, DH93, and DH103 were also used to make a cross with M564. So, two groups of complementary heterozygous genotypes were obtained, namely *TaPpm1a/1d//TaPpb1a/1b* and *TaPpm1a/1b//TaPpb1a/1b* including 3 hybrids separately (Fig. S1). Mature seeds of these 6 F_1 plants all emerged purple color, though obviously lighter than that of ND3753 (Fig. 1b and c). This verified that the interaction of two transcription factors recovered anthocyanin biosynthesis but showed a certain dosage effect. Since the cross DH41/DH33 bared relatively deeper color seeds, it was chosen for further studies. Observation color change of seeds at 12, 17, 22, 27, and 32 DAP showed that DH41, DH33, and LX99 did not accumulate anthocyanins during the whole development of grains, whereas ND3753 began to emerge purple color at 17 DAP while DH41/DH33 at 22 DAP (Fig. 3). Correspondingly, measurement of grain anthocyanin content revealed that anthocyanin was not detected in DH41, DH33, and LX99, while its content reached 1.35 mg/100 g in ND3753 at 17 DAP and 0.25 mg/100 g in DH41/DH33 at 22 DAP. Moreover, grains of the final stage of ND3753 and DH41/DH33 contained anthocyanins of 29.74 mg/100 g and 22.26 mg/100 g, respectively (Table 1). In summary, heterozygous complementation of *TaPpm1* and *TaPpb1* could recover anthocyanin biosynthesis, however, its efficacy at this status was significantly lower than that of homozygous.

Relative expression levels of *TaPpm1* and *TaPpb1* in different genotypes

To explore the reason why ND3753 accumulated anthocyanins earlier and quicker than DH41/DH33, the qRT-PCR was used to determine the expression levels of *TaPpm1* and *TaPpb1* in developing grains of different genotypes. LX99 carried double recessive alleles on both loci while ND3753 carried dominant ones, it isn't unexpected that the two genes remained very low- or un-expression in LX99 while they were highly expressed in ND3753. *TaPpm1* expressed highly in ND3753 at the middle grain

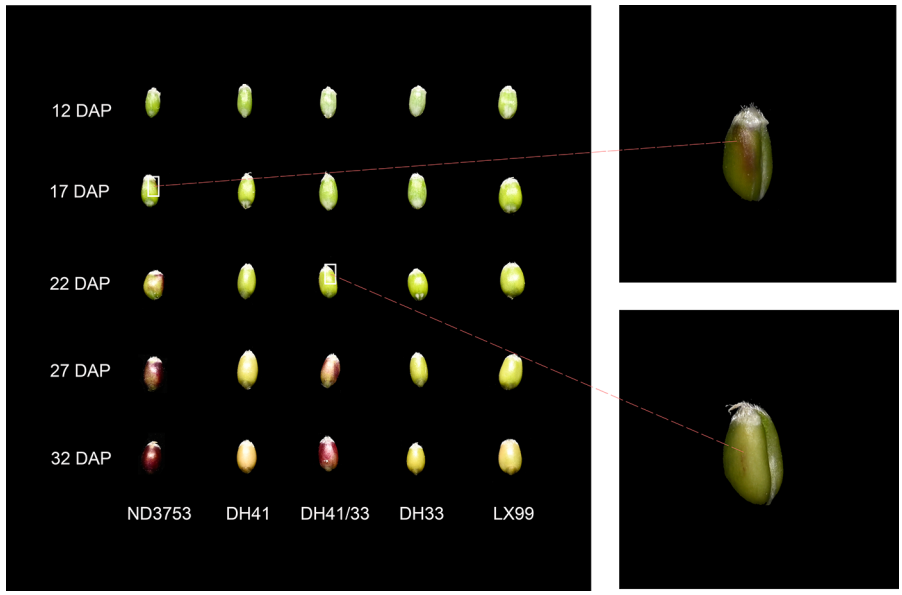

Fig. 3 Phenotypic observation on pericarp color of different genotypes of regulatory genes at 12, 17, 22, 27, and 32 DAP (days after pollination) during grain development stages

Table 1 Determination of anthocyanin content (mg/100 g) in grains of different genotypes of regulatory genes at five grain development stages

	12 DAP	17 DAP	22 DAP	27 DAP	32 DAP
ND3753	0	1.35 ± 0.01^{a}	2.55 ± 0.07^{a}	18.92 ± 0.09^{a}	29.74 ± 0.15^{a}
DH41	0	0^{b}	0^{c}	0^{c}	0^{c}
DH41/DH33	0	0^{b}	0.25 ± 0.05^{b}	12.35 ± 0.10^{b}	22.26 ± 0.11^{b}
DH33	0	0^{b}	0^{c}	0^{c}	0^{c}
LX99	0	0^{b}	0^{c}	0^{c}	0^{c}

Data are shown as means ± SD of three biological replicates. Duncan's multiple range tests are used for statistical analysis, different letters in the same column indicate significant differences ($P < 0.05$)

development stages (17 and 22 DAP) and decrease rapidly at later stages (27 and 32 DAP), whereas *TaPpb1* maintained high expression through middle and later stages (Fig. 4). It is also reasonable that *TaPpm1* in DH33 and *TaPpb1* in DH41 remained low- or un-expression because of corresponding double recessive alleles separately carried in them. What is interesting is that the expression patterns of these two genes in other statuses were very different. Dominant alleles of *TaPpm1* exited all in DH41, DH41/DH33, and ND3753, but its expression levels in DH41 and DH41/DH33 were significantly lower than in ND3753 at 17 and 22 DAP. However, when its expression decreased at later stages in ND3753, its expression increased in DH41 and DH41/DH33 and reached a relatively higher level in DH41/DH33 at 32 DAP (Fig. 4a). On the contrary, *TaPpb1* in DH41/DH33 and DH33, despite its status of single or double dominant alleles, expressed quite consistent with that of ND3753 at all stages (Fig. 4b). In other words, *TaPpb1* can express persistently once its dominant allele exists, whereas expression of *TaPpm1* is affected by its own and *TaPpb1* dominant allele status. These results implied that *TaPpm1* but not *TaPpb1* should be the key factor that resulted in anthocyanin reduction in the heterozygous genotype of purple-grained wheat.

Relative expression levels of structural genes of anthocyanin biosynthesis

TaPpm1 and *TaPpb1* should be through regulating structural genes on the anthocyanin biosynthesis pathway to control anthocyanin accumulation in

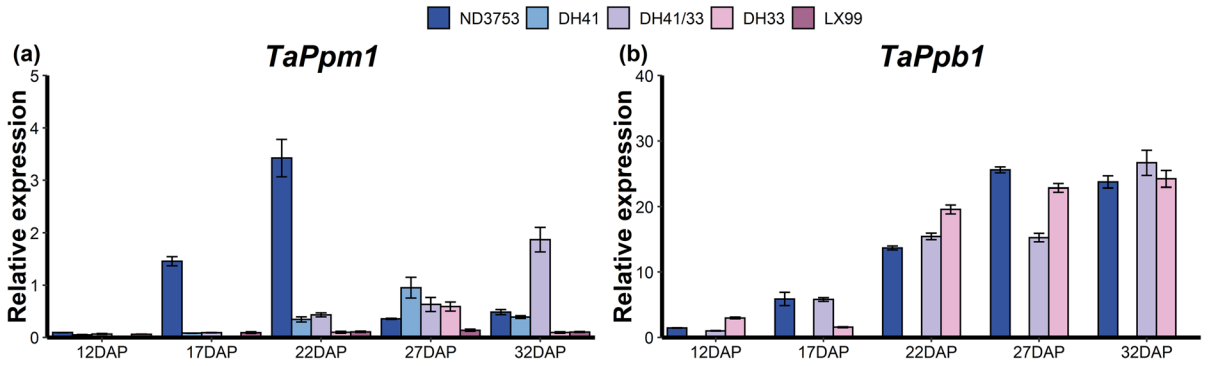

Fig. 4 Relative expression levels of *TaPpm1* and *TaPpb1* in different combinations of genotypes on different days after pollination. **a** Relative expression levels of *TaPpm1* at 12, 17, 22, 27, 32 DAP. **b** Relative expression levels of *TaPpb1* at 12, 17, 22, 27, 32 DAP. Data are shown as means ± SD of three biological replicates

purple-grained wheat. So, expression of structural genes including *CHS*, *CHI*, *F3H*, *F3'H*, *F3'5'H*, *DFR*, and *ANS* was detected during grain development in different genotypes. Because anthocyanin accumulated only in grains of ND3753 and DH41/DH33, target genes should be those expressed higher at key grain development stages in these two genotypes than that in others without anthocyanin accumulation. Besides, it is also crucial that their expression patterns should correspond with that of *TaPpm1* which was the key factor that caused the difference in anthocyanin accumulation. Upon these criteria, *CHI* and *F3H* could not be target genes, because their expression levels in some non-purple-grained genotypes were significantly higher than that in ND3753 and DH41/DH33 at 17 and/or 22 DAP stages (Fig. 5b and c). The expression level of *CHS* in ND3753 was ever-increasing through grain development stages but at 22 DAP was significantly lower than that in DH41/DH33 (Fig. 5a), whereas *F3'5'H* maintained lower expression during 12–27 DAP in all genotypes but simultaneously increased sharply at 32 DAP in ND3753 and DH41/DH33 (Fig. 5e). So, *CHS* and *F3'5'H* also should not be target genes according to their inconsistency expression patterns with that of *TaPpm1*. On the other hand, expression patterns of *F3'H*, *DFR*, and *ANS* met the criteria of target genes quite well. They all expressed significantly higher in purple-grained genotypes than in nonpurple ones at all grain development stages except *ANS* at a few stages, and between purple-grained genotypes their expression levels in heterozygous were significantly lower than in homozygous at most stages (Fig. 5d, f and g).

Discussion

The formation of anthocyanins is regulated by a complex formed by MYB, bHLH, and WD40 transcription factors. In natural materials, WD40 transcription factors have complex functions and have not yet been studied in-depth, and they can all be expressed normally (Strygina and Khlestkina 2019). Therefore, there were two complementary dominant genes controlling the purple pericarp of wheat in previous mapping studies (Tereshchenko et al. 2012; Jiang et al. 2018). In our research, the anthocyanins could not be detected in the pericarp of genotypes with a single transcription factor across the five stages of grain development. The purple appeared only when two transcription factors were present at the same time (Fig. 1 and 3), which was consistent with previous studies. It is worth mentioning that for these two transcription factors, we used two white pericarp lines to obtain purple pericarp seeds of F_1 plants through genetic complementarity (Fig. 1b and c), which was consistent with the principle of heterosis utilization. As an extremely important genetic improvement technique in current production practice, heterosis plays an important role in genetic breeding (Jiang et al. 2017). Previous research has shown that gene differential expression between the hybrids and parents is one of the molecular sources of plant heterosis. The differentially expressed genes between the parents

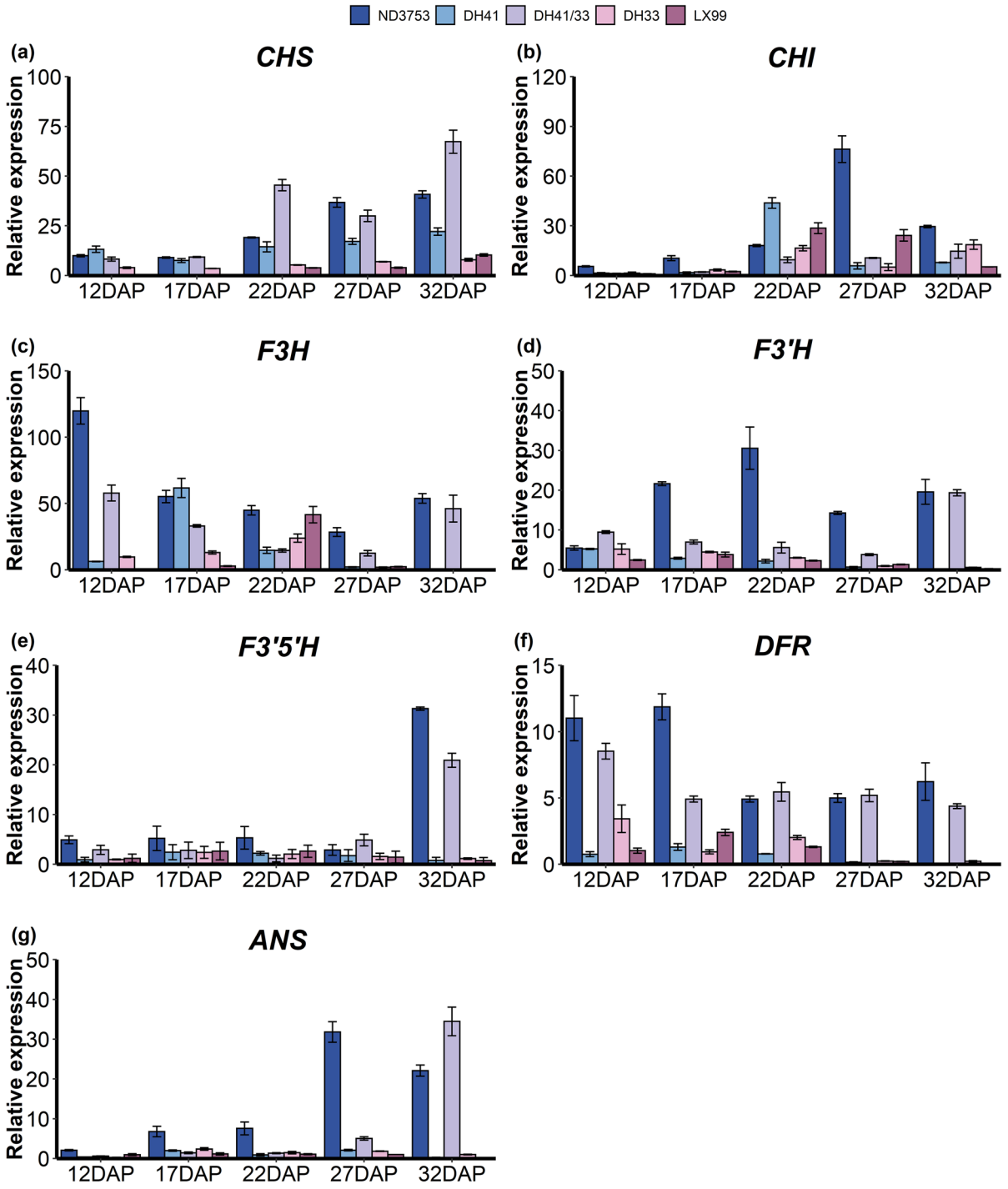

Fig. 5 Relative expression levels of seven structural genes in different genotypes of regulatory genes on different days after pollination. Data are shown as means ± SD of three biological replicates

and hybrid at the transcriptome level in early maize root tissue development mainly show a complementary expression pattern (Paschold et al. 2012). Similar results were reported in the current study, in which the expression of structural genes related to anthocyanin biosynthesis in the seeds of F_1 plants was higher than that of the two parents, resulting in the pericarp of heterozygote DH41/DH33 appearing purple (Fig. 3 and 5, Table 1).

It is difficult for breeders to make the crossing between germplasms with white grains to get progenies with purple grains. A previous study has found that in 102 samples of common hexaploid wheat, 14 materials with purple pericarp contained *TaMYC1p* (*TaPpb1a*), and the remaining 88 materials with white pericarp all carried *TaMYC1w* (*TaPpb1b*) (Zong et al. 2017). Another study used the developed marker to detect the allelic variation of *TaPpm1* and *TaPpb1* in 34 varieties of wheat. The four purple-grain varieties all contained *TaPpm1a/TaPpb1a*, while the genotypes of other non-purple-grain varieties were *TaPpm1a/TaPpb1b*, *TaPpm1b/TaPpb1b*, *TaPpm1c/TaPpb1b*, and *TaPpm1d/TaPpb1b* respectively (Jiang et al. 2018). It seems that most varieties containing *TaPpb1b* had white pericarp and thus this allele had epistasis effects on grain color. Therefore, even if these white pericarp materials were crossed with white pericarp carrying the *TaPpm1a*, their progeny couldn't produce purple pericarp either. In this study, we successfully obtained purple grains progeny by the selection of two white pericarp DH lines carrying *TaPpm1a* and *TaPpb1a* through genotyping (Fig. 1b and c, 2). At present, the use of assisted selection with markers closely linked to important agronomic traits to achieve multi-gene aggregation breeding has become the mainstream of breeding development. For example, molecular markers were used to aggregate two QTLs for resistance to Fusarium head blight (FHB) to accelerate the improvement of disease-resistant and high-yielding varieties (Salameh et al. 2011). In addition, the molecular marker-assisted selection was successfully used to pyramid excellent genes that control different traits in single plants with both disease resistance and high quality (Zheng et al. 2020). Results from this study were in line with these studies and confirmed the valuable roles of molecular markers in increasing the breeding efficiency of traits that was hard to select via conventional breeding.

Since the pericarp is developed from the ovary wall, the heterozygous material for regulatory genes used in this experiment was obtained from the seeds of F_1 plants. In the current research, the DH population hybridization showed that the anthocyanin content in the purple pericarp of the heterozygous genotype of the two regulatory genes was lower than that of the homozygous genotype, and there was a certain color gradient (Fig. 1 and 3, Table 1). This may be due to differences in the genetic background of the DH lines, which showed lighter pericarp color than ND3753. Furthermore, the diversity in phenotype may be related to the difference in gene expression (Veitia et al. 2008; Schadt et al. 2003). For instance, the maximum transcriptional levels of the large and small alleles of *fw2.2*, which is associated with fruit weight, differ by about one week during tomato fruit size development, and changes in the duration of gene expression and differences in total transcriptional levels caused significant differences in fruit weight (Cong et al. 2002). In rice, *GS5* expression levels in two near-isogenic lines (NILs) are measured at different times and in tissues. It is found that such expression differences are consistent with grain width and filling stage, and individual plants with more sustained high expression levels can produce wider grains (Li et al. 2011). The expression time and peak period of the cell cycle-related genes in cucumber affect the replication level and cell division of cucumber fruit, thereby affecting fruit development (Fu et al. 2010). Likewise, this study also found that there may be dosage effects of gene expression on anthocyanin biosynthesis. The expression time of the regulatory genes of the DH41/DH33 in the anthocyanin biosynthesis pathway was generally later than that of ND3753, and the overall expression was lower than ND3753 during grain development, as well as the same trend was observed among the structural genes (Fig. 4 and 5). As a result, the different expression amount of these genes may cause different levels of secondary metabolites involved in anthocyanin synthesis, which then eventually leads to less accumulation of anthocyanin content between grains of DH41/DH33 than those of ND3753.

A previous study has used NILs to analyze the specific expression of anthocyanin synthesis structural genes *CHI* and *F3H* in the pericarp by using different combinations of *Pp* alleles by qRT-PCR. The results showed that one of the genes was sufficient to

activate the transcription of *CHI*, while the activation of *F3H* depended on the coexistence of *Pp-D1* and *Pp3* (Gordeeva et al. 2015). In addition, *Ant1* encoding an R2R3-MYB TF and *Ant2* encoding a bHLH TF form a complex to activate anthocyanin synthesis in barley lemma and pericarp by affecting the expression of *F3'H* and *ANS* (Gordeeva et al. 2019). Furthermore, A model of *C-S-A* gene regulation of rice hull pigmentation has been proposed, in which *C1* (an R2R3-MYB TF) interacts with *S1* (a bHLH TF) and activates the expression of *A1* (*OsDFR*), giving rice hull purple color (Sun et al. 2018). By contrast, with the increased expression of *TaPpm1* and *TaPpb1* in the purple pericarp, all the structural genes in the anthocyanin biosynthesis pathway were expressed in the purple pericarp, and the expression levels of *CHS*, *F3'H*, *F3'5'H*, *DFR*, and *ANS* were significantly higher than those in white pericarp except for *CHI* and *F3H*. *CHS*, *CHI*, and *F3H* were expressed in the white pericarp, while *F3'H*, *F3'5'H*, *DFR*, and *ANS* were very low or almost not expressed in the white pericarp (Fig. 5). Among them, CHS and CHI are key enzymes in the metabolic pathway of flavonoids, in addition to anthocyanins, they are also involved in the biosynthesis of multiple pigment pathways such as proanthocyanidins (PA) and flavonols (Koes et al. 2005; Petroni and Tonelli 2011; Khlestkina et al. 2015). Nevertheless, the expression levels of *TaPpm1* were highly correlated with the expression levels of *F3'H*, *DFR*, and *ANS* in grains (Fig. 5d, f and g). Therefore, we speculated that the co-expression of *TaPpm1* and *TaPpb1* in wheat purple pericarp mainly regulated *F3'H*, *DFR*, and *ANS*, whereas more methods are needed to verify their interaction with these regulatory genes.

Conclusion

The dosage effect of purple grain color in wheat should be due to the different expression of genes involving anthocyanin biosynthesis in different genotypes. This study demonstrated that out of two regulatory genes, only *TaPpm1* was decreased expression at early grain developing stages, and consequently down regulated structural genes *F3'H*, *DFR*, and *ANS* and finally reduced anthocyanin accumulation in grains of the heterozygous genotype.

Author contributions All authors contributed to the study conception and design. MY supervised and designed the study; ST provided the DH population; CB and CW completed the experiment; JL and ST assisted in the experiment; CB and CW analyzed the data and wrote the first draft; JL, YZ, JM, and MY assisted in revising the first draft. All authors read and approved the final manuscript.

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Declarations

Conflict of interest The authors have no relevant financial or non-financial interests to disclose.

References

Chen SY, Wang SC (2019) GLABRA2, a common regulator for epidermal cell fate determination and anthocyanin biosynthesis in *Arabidopsis*. Int J Mol Sci. https://doi.org/10.3390/ijms20204997

Cong B, Liu JP, Tanksley SD (2002) Natural alleles at a tomato fruit size quantitative trait locus differ by heterochronic regulatory mutations. P Natl Acad Sci Usa 99(21):13606–13611. https://doi.org/10.1073/pnas.172520999

Devi KD, Punyarani K, Singh NS, Devi HS (2013) An efficient protocol for total DNA extraction from the members of order Zingiberales-suitable for diverse PCR based downstream applications. Springer plus 2(1):669. https://doi.org/10.1186/2193-1801-2-669

Dobrovolskaya O, Arbuzova VS, Lohwasser U, Roder MS, Borner A (2006) Microsatellite mapping of complementary genes for purple grain colour in bread wheat (*Triticum aestivum* L.). Euphytica 150(3):355–364. https://doi.org/10.1007/s10681-006-9122-7

Dong NQ, Sun YW, Guo T, Shi CL, Zhang YM, Kan Y, Xiang YH, Zhang H, Yang YB, Li YC, Zhao HY, Yu HX, Lu ZQ, Wang Y, Ye WW, Shan JX, Lin HX (2020) UDP-glucosyltransferase regulates grain size and abiotic stress tolerance associated with metabolic flux redirection in rice. Nat Commun. https://doi.org/10.1038/s41467-020-16403-5

Fan XP, Fan BH, Wang YX, Yang WC (2016) Anthocyanin accumulation enhanced in *Lc*-transgenic cotton under light and increased resistance to bollworm. Plant Biotechnol Rep 10(1):1–11. https://doi.org/10.1007/s11816-015-0382-3

Ficco D, De Simone V, Colecchia SA, Pecorella I, Platani C, Nigro F, Finocchiaro F, Papa R, De Vita P (2014) Genetic variability in anthocyanin composition and nutritional properties of blue, purple, and red bread (*Triticum aestivum* L.) and Durum (*Triticum turgidum* L. ssp. *turgidum* convar. *durum*) wheats. J Agr Food Chem 62(34):8686–8695. https://doi.org/10.1021/jf5003683

Fu FQ, Mao WH, Shi K, Zhou YH, Yu JQ (2010) Spatio-temporal changes in cell division, endoreduplication and expression of cell cycle-related genes in pollinated and

plant growth substances-treated ovaries of cucumber. Plant Biol 12(1):98–107. https://doi.org/10.1111/j.1438-8677.2009.00203.x

Gaudin ACM, McClymont SA, Soliman SSM, Raizada MN (2014) The effect of altered dosage of a mutant allele of Teosinte branched 1 (*tb1-ref*) on the root system of modern maize. Bmc Genet 15(23):14–2014. https://doi.org/10.1186/1471-2156-15-23

Gordeeva EI, Shoeva OY, Khlestkina EK (2015) Marker-assisted development of bread wheat near-isogenic lines carrying various combinations of purple pericarp (*Pp*) alleles. Euphytica 203(2):469–476. https://doi.org/10.1007/s10681-014-1317-8

Gordeeva EI, Glagoleva AY, Kukoeva TV, Khlestkina EK, Shoeva OY (2019) Purple-grained barley (*Hordeum vulgare* L.): marker-assisted development of NILs for investigating peculiarities of the anthocyanin biosynthesis regulatory network. Bmc Plant Biol. https://doi.org/10.1186/s12870-019-1638-9

Grotewold E, Drummond BJ, Bowen B, Peterson T (1994) The myb-homologous *P* gene controls phlobaphene pigmentation in maize floral organs by directly activating a flavonoid biosynthetic gene subset. Cell 76(3):543–553. https://doi.org/10.1016/0092-8674(94)90117-1

Hatier J, Clearwater MJ, Gould KS (2013) The functional significance of black-pigmented leaves: photosynthesis, photoprotection and productivity in *Ophiopogon planiscapus* 'Nigrescens.' PLoS ONE. https://doi.org/10.1371/journal.pone.0067850

Himi E, Nisar A, Noda K (2005) Colour genes (*R* and *Rc*) for grain and coleoptile upregulate flavonoid biosynthesis genes in wheat. Genome 48(4):747–754. https://doi.org/10.1139/G05-026

Hu JP, Anderson B, Wessler SR (1996) Isolation and characterization of rice *R* genes: Evidence for distinct evolutionary paths in rice and maize. Genetics 142(3):1021–1031. https://doi.org/10.1007/BF00054630

Isaak CK, Petkau JC, Blewett H, Karmin O, Siow YL (2017) Lingonberry anthocyanins protect cardiac cells from oxidative-stress-induced apoptosis. Can J Physiol Pharm 95(8):904–910. https://doi.org/10.1139/cjpp-2016-0667

Jiang Y, Schmidt RH, Zhao YS, Reif JC (2017) A quantitative genetic framework highlights the role of epistatic effects for grain-yield heterosis in bread wheat. Nat Genet 49(12):1741. https://doi.org/10.1038/ng.3974

Jiang WH, Liu TX, Nan WZ, Jeewani DC, Niu YL, Li CL, Wang Y, Shi X, Wang C, Wang JH, Li Y, Gao X, Wang ZH (2018) Two transcription factors TaPpm1 and TaPpb1 co-regulate anthocyanin biosynthesis in purple pericarps of wheat. J Exp Bot 69(10):2555–2567. https://doi.org/10.1093/jxb/ery101

Khlestkina EK (2013) Genes determining the coloration of different organs in wheat. Russ J Genet: Appl Res 3(1):54–65. https://doi.org/10.1134/S2079059713010085

Khlestkina EK, Shoeva OY, Gordeeva EI (2015) Flavonoid biosynthesis genes in wheat. Russ J Genet: Appl Res 5(3):268–278. https://doi.org/10.1134/s2079059715030077

Koes R, Verweij W, Quattrocchio F (2005) Flavonoids: a colorful model for the regulation and evolution of biochemical pathways. Trends Plant Sci 10(5):236–242. https://doi.org/10.1016/j.tplants.2005.03.002

Kong XC, Wang F, Geng SF, Guan JT, Tao S, Jia ML, Sun GL, Wang ZY, Wang K, Ye XG, Ma J, Liu DC, Wei YM, Zheng YL, Fu XD, Mao L, Lan XJ, Li AL (2022) The wheat *AGL6*-like MADS-box gene is a master regulator for floral organ identity and a target for spikelet meristem development manipulation. Plant Biotechnol J 20(1):75–88. https://doi.org/10.1111/pbi.13696

Li YB, Fan CC, Xing YZ, Jiang YH, Luo LJ, Sun L, Shao D, Xu CJ, Li XH, Xiao JH, He YQ, Zhang QF (2011) Natural variation in *GS5* plays an important role in regulating grain size and yield in rice. Nat Genet 43(12):1266. https://doi.org/10.1038/ng.977

Li X, He YM, Xie CM, Zu YQ, Zhan FD, Mei XY, Xia Y, Li Y (2018) Effects of UV-B radiation on the infectivity of Magnaporthe oryzae and rice disease-resistant physiology in Yuanyang terraces. Photoch Photobio Sci 17(1):8–17. https://doi.org/10.1039/c7pp00139h

Li XL, Lu X, Wang XH, Peng Q, Zhang MS, Ren MJ (2020) Biotic and abiotic stress-responsive genes are stimulated to resist drought stress in purple wheat. J Integr Agr 19(1):33–50. https://doi.org/10.1016/S2095-3119(19)62659-6

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(T)(-Delta Delta C) method. Methods 25(4):402–408. https://doi.org/10.1006/meth.2001.1262

Ludwig SR, Habera LF, Dellaporta SL, Wessler SR (1989) *Lc*, a member of the maize *R* gene family responsible for tissue-specific anthocyanin production, encodes a protein similar to transcriptional activators and contains the myc-homology region. P Natl Acad Sci USA 86(18):7092–7096. https://doi.org/10.1073/pnas.86.18.7092

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(3):1252–1268. https://doi.org/10.1104/pp.106.094425

Martin C, Gerats T (1993) Control of pigment biosynthesis genes during petal development. Plant Cell 5(10):1253–1264. https://doi.org/10.1105/tpc.5.10.1253

Mol J, Grotewold E, Koes R (1998) How genes paint flowers and seeds. Trends Plant Sci 3(6):212–217. https://doi.org/10.1016/S1360-1385(98)01242-4

Mrkvicova E, Pavlata L, Karasek F, Stastnik O, Dolezalova E, Trojan V, Vyhnanek T, Hrivna L, Holeksova V, Mares J, Brabec T, Horky P, Ruttkay-Nedecky B, Adam V, Kizek R (2016) The influence of feeding purple wheat with higher content of anthocyanins on antioxidant status and selected enzyme activity of animals. Acta Vet Brno 85(4):371–376. https://doi.org/10.2754/avb201685040371

Nakabayashi R, Yonekura-Sakakibara K, Urano K, Suzuki M, Yamada Y, Nishizawa T, Matsuda F, Kojima M, Sakakibara H, Shinozaki K, Michael AJ, Tohge T, Yamazaki M, Saito K (2014) Enhancement of oxidative and drought tolerance in Arabidopsis by overaccumulation of antioxidant flavonoids. Plant J 77(3):367–379. https://doi.org/10.1111/tpj.12388

Paschold A, Jia Y, Marcon C, Lund S, Larson NB, Yeh CT, Ossowski S, Lanz C, Nettleton D, Schnable PS,

Hochholdinger F (2012) Complementation contributes to transcriptome complexity in maize (*Zea mays* L.) hybrids relative to their inbred parents. Genome Res 22(12):2445–2454. https://doi.org/10.1101/gr.138461.112

Peiffer DS, Wang LS, Zimmerman NP, Ransom B, Carmella SG, Kuo CT, Chen JH, Oshima K, Huang YW, Hecht SS, Stoner GD (2016) Dietary consumption of black raspberries or their anthocyanin constituents alters innate immune cell trafficking in esophageal cancer. Cancer Immunol Res 4(1):72–82. https://doi.org/10.1158/2326-6066.CIR-15-0091

Petroni K, Tonelli C (2011) Recent advances on the regulation of anthocyanin synthesis in reproductive organs. Plant Sci 181(3):219–229. https://doi.org/10.1016/j.plantsci.2011.05.009

Quattrocchio F, Verweij W, Kroon A, Spelt C, Mol J, Koes R (2006) *PH4* of petunia is an R2R3 MYB protein that activates vacuolar acidification through interactions with basic-helix-loop-helix transcription factors of the anthocyanin pathway. Plant Cell 18(5):1274–1291. https://doi.org/10.1105/tpc.105.034041

Reddy VS, Scheffler BE, Wienand U, Wessler SR, Reddy AR (1998) Cloning and characterization of the rice homologue of the maize *C1* anthocyanin regulatory gene. Plant Mol Biol 36:497–498. https://doi.org/10.1023/A:1017106913186

Saitoh K, Onishi K, Mikami I, Thidar K, Sano Y (2004) Allelic diversification at the *C* (*OsC1*) locus of wild and cultivated rice: Nucleotide changes associated with phenotypes. Genetics 168(2):997–1007. https://doi.org/10.1534/genetics.103.018390

Salameh A, Buerstmayr M, Steiner B, Neumayer A, Lemmens M, Buerstmayr H (2011) Effects of introgression of two QTL for fusarium head blight resistance from Asian spring wheat by marker-assisted backcrossing into European winter wheat on fusarium head blight resistance, yield and quality traits. Mol Breed 28(4):485–494. https://doi.org/10.1007/s11032-010-9498-x

Schadt EE, Monks SA, Drake TA, Lusis AJ, Che N, Colinayo V, Ruff TG, Milligan SB, Lamb JR, Cavet G, Linsley PS, Mao M, Stoughton RB, Friend SH (2003) Genetics of gene expression surveyed in maize, mouse and man. Nature 422(6929):297–302. https://doi.org/10.1038/nature01434

Strygina KV, Khlestkina EK (2019) Structural and functional organization and evolution of the *WD40* genes involved in the regulation of flavonoid biosynthesis in the Triticeae Tribe. Russ J Genet+ 55(11):1398–1405. https://doi.org/10.1134/S1022795419110152

Sun XM, Zhang ZY, Chen C, Wu W, Ren NN, Jiang CH, Yu JP, Zhao Y, Zheng XM, Yang QW, Zhang HL, Li JJ, Li ZC (2018) The *C-S-A* gene system regulates hull pigmentation and reveals evolution of anthocyanin biosynthesis pathway in rice. J Exp Bot 69(7):1485–1498. https://doi.org/10.1093/jxb/ery001

Tereshchenko OY, Gordeeva EI, Arbuzova VS, Borner A, Khlestkina EK (2012) The D genome carries a gene determining purple grain colour in wheat. Cereal Res Commun 40(3):334–341. https://doi.org/10.1556/CRC.40.2012.3.2

Tian S, Zhang M, Li J, Wen S, Bi C, Zhao H, Wei C, Chen Z, Yu J, Shi X, Liang R, Xie C, Li B, Sun Q, Zhang Y, You M (2021) Identification and validation of stable quantitative trait loci for SDS-sedimentation volume in common wheat (*Triticum aestivum* L.). Front Plant Sci 12:747775. https://doi.org/10.3389/fpls.2021.747775

Veitia RA, Bottani S, Birchler JA (2008) Cellular reactions to gene dosage imbalance: genomic, transcriptomic and proteomic effects. Trends Genet 24(8):390–397. https://doi.org/10.1016/j.tig.2008.05.005

Yuan YX, Chiu LW, Li L (2009) Transcriptional regulation of anthocyanin biosynthesis in red cabbage. Planta 230(6):1141–1153. https://doi.org/10.1007/s00425-009-1013-4

Zheng J, Wu H, Zhu HB, Huang CY, Liu C, Chang YS, Kong ZC, Zhou ZH, Wang GW, Lin YJ, Chen H (2019) Determining factors, regulation system, and domestication of anthocyanin biosynthesis in rice leaves. New Phytol 223(2):705–721. https://doi.org/10.1111/nph.15807

Zheng WJ, Li S, Liu ZH, Zhou Q, Feng YR, Chai SC (2020) Molecular marker assisted gene stacking for disease resistance and quality genes in the dwarf mutant of an elite common wheat cultivar Xiaoyan22. Bmc Genet. https://doi.org/10.1186/s12863-020-00854-2

Zhu QL, Sui SZ, Lei XH, Yang ZF, Lu K, Liu GD, Liu YG, Li MY (2015) Ectopic expression of the coleus R2R3 MYB-type proanthocyanidin regulator gene *SsMYB3* alters the flower color in transgenic Tobacco. PLoS ONE. https://doi.org/10.1371/journal.pone.0139392

Zimmermann IM, Heim MA, Weisshaar B, Uhrig JF (2004) Comprehensive identification of *Arabidopsis thaliana* MYB transcription factors interacting with R/B-like BHLH proteins. Plant J 40(1):22–34. https://doi.org/10.1111/j.1365-313X.2004.02183.x

Zong Y, Xi XY, Li SM, Chen WJ, Zhang B, Liu DC, Liu BL, Wang DW, Zhang HG (2017) Allelic variation and transcriptional isoforms of wheat *TaMYC1* gene regulating anthocyanin synthesis in pericarp. Front Plant Sci. https://doi.org/10.3389/fpls.2017.01645