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Cloning of *GzABI5-3A3* Gene of 'Guizimai 1' and Functional Analysis in Tobacco

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Abstract *ABI5* plays an important role in many biological processes such as seed dormancy and germination, growth and development, anthocyanin synthesis and response to stress. A large number of studies have shown that *ABI5* is involved in seed dormancy and germination, while few studies have shown that *ABI5* is involved in anthocyanin synthesis. In order to explore the involvement of *ABI5* transcription factors in the regulation of wheat anthocyanin synthesis, the full cDNA length of Gz*ABI5-3A3* was amplified from a color wheat variety 'Guizimai 1'. Gz*ABI5-3A3* may be involved in biological processes regulating plant growth, photosynthesis, flowering, seed germination and anthocyanin accumulation. Phylogenetic tree analysis showed that Gz*ABI5-3A3* was homologous to Ta*ABI5D-SH-31*, Ta*ABI5D-SH-23* and Ta*ABI5D-SW-23* in wheat. In this study, the overexpression vector of Gz*ABI5-3A3* gene PBI121-Gz*ABI5-3A3* was further constructed for tobacco genetic transformation. Eight tobacco transgenic lines were obtained by genetic transformation. Studies on Gz*ABI5-3A3* overexpressed transgenic lines showed that the anthocyanin content in seedling leaves of transgenic tobacco lines L4, L7 and L15 was significantly lower than that of the wild type, and the expression levels of anthocyanin synthesis pathways of structural genes Nt*PAL*, Nt*DFR*, Nt*ANS* and Nt*CHS* were significantly decreased. The results showed that Gz*ABI5-3A3* could negatively regulate anthocyanin synthesis by regulating the expression of structural genes in anthocyanin synthesis pathway. This study provides a research basis for the subsequent research on the molecular mechanism of Gz*ABI5-3A3* regulating anthocyanin synthesis.
Keywords 'Guizimai 1'; Gz*ABI5-3A3*; Gene clone; Tobacco genetic transformation

Basic domain leucine zipper (bzip) transcription factors are the most conserved and widely distributed transcription factors in eukaryotes, which exists in most eukaryotic cells (Zhang et al., 2017; Cui et al., 2019). In plants, according to the structure and function of bzip transcription factors, the family can be divided into 10 subfamilies (namely A, B, C, D, E, F, G, H, I and S) (Yang et al., 2009), and its recognition core sequence is cis-acting elements containing ACGT, such as CACGTG (G box), GACGTC (C box), TACGTA (A box) (Ali et al., 2016). It was found that the promoter regions of some genes induced by light or abscisic acid (ABA) contain the above elements (Zhang, 2018). ABI5 (Abscisic acid insensitive 5) subfamily is a bzip transcription factor induced by ABA. It is found that it is closely related to stress resistance of plant (Finkelstein and Lynch, 2000; Zou et al., 2008). *ABI5* can combine with specific cis-acting elements to form a regulatory network to improve the adaptability of plants to environmental stress and enable them to regulate their growth under stress. *ABI5* plays an important role in many biological processes such as seed dormancy and germination, growth and plant response to stress. Miura et al. (2009) found that *ABI5* inhibited seed germination and the sensitivity of seedling primary roots to ABA. Ibarra et al. (2015) found that *RGL2* promotes seed secondary dormancy by regulating *ABI5* expression and ABA synthesis. Guan et al. (2014) showed that *ABI5* is an important factor regulating ABA mediated seed germination and growth inhibition after germination. Yang et al. (2016) found that in *Arabidopsis thaliana* (Linn.) Heynh., the decrease of sensitivity of *BZR1-1D* mutant to ABA is mediated by *ABI5*. Liao et al. (2016) showed that *ABI5* is involved in regulating the response of *Arabidopsis thaliana* to pathogens and abscisic acid. In addition, *ABI5* is also involved in regulating anthocyanin accumulation. Hoth et al. (2010) found that *ABI5* in *Arabidopsis thaliana* plays a role in sucrose induced anthocyanin accumulation. After 3% sucrose treatment, the

anthocyanin accumulation of ABI5-4 mutant increased, which was related to the increased expression of At*SUC1*. It has been found that ABA signal induced anthocyanin accumulation in *Arabidopsis thaliana* seedlings, which may be achieved through the synergistic effect of *ABI5* and MBW complex. It can be seen that *ABI5* can regulate the accumulation of anthocyanins by regulating the expression of other anthocyanin related genes, but its mechanism needs to be further studied.

In *Triticum aestivum* L., *ABI5* plays an important regulatory role in the biological processes of ear germination (Zhou et al., 2017) and seed dormancy and germination (Sun, 2016; Utsugi et al., 2020). Utsugi et al. (2020) obtained Ta*ABI5* full-length linked overexpression vector from *Triticum aestivum* by amplification to transform *Arabidopsis thaliana*. It was found that the seed dormancy of *Arabidopsis thaliana* overexpression line was stronger than that of wild type. 'Guizimai 1' with purple grain was selected by Guizhou Branch of National Wheat Improvement Center through distant hybridization of *Aegilops tauschii*, *Triticum ventricosum* and *Triticum turgidum* L. *var. durum*. It has the characteristics of high yield, strong stress resistance and rich anthocyanins (Xu et al., 2018). At present, there is no relevant report that *ABI5* involves in the regulation of anthocyanin accumulation of *Triticum aestivum*.

Therefore, we analyzed the biological information and expression pattern of Gz*ABI5-3A3* in 'Guizimai 1', and further verified the overexpression transgenic tobacco lines in this study, so as to provide a research basis for further study on the molecular mechanism of *ABI5* transcription factor regulating anthocyanin synthesis.

1 Results and Analysis

1.1 Construction of overexpression vector of Gz*ABI5-3A3* gene of 'Guizimai 1'

Using the cDNA extracted from the grain of 'Guizimai 1' 25 days after flowering as the template, the full length of Gz*ABI5-3A3* sequence was amplified by PCR. A target fragment of more than 1 000 bp was obtained by 1% agarose gel electrophoresis (Figure 1) and connected to the overexpression vector pBI121. The plasmid was sent to the company for sequencing. The sequencing results showed that the amplified sequence was consistent with the sequence of Gz*ABI5-3A3*.

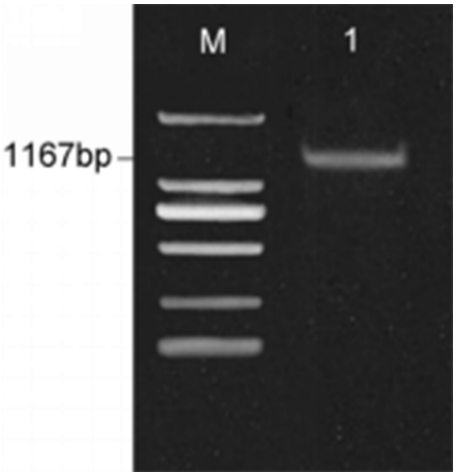

Figure 1 PCR analysis of Gz*ABI5-3A3*
Note: M: DL2000 Marker; 1: Gz*ABI5-3A3* amplified product

1.2 Primary structure and secondary structure of protein encoded by Gz*ABI5-3A3* gene

Using InterProScan to analyze the protein domain of Gz*ABI5-3A3*, it was found that its protein belongs to bzip gene family and contains a bzip conserved domain, which is located from 296th amino acid to 362nd amino acid. The protein characteristics were analyzed online by ProtPara. Its molecular formula was $C_{1803}H_{2885}N_{533}O_{575}S_{33}$, molecular weight was 42 286.95, isoelectric point was 5.79, instability index was 56.53, fat index was 62.56, including 48 negatively charged residues and 39 positively charged residues. By analyzing its hydrophilicity and hydrophobicity through ProtScale, the analysis results showed that the average value of protein hydrophobicity is -0.501, and the protein instability coefficient is larger than 40, indicating that the protein is in an unstable state,

and its average hydrophobicity value is less than 0, indicating that the protein is a hydrophilic protein (Figure 2). Using SOPMA tool to analyze the secondary structure, it was found that the secondary structure (Figure 3) is mainly α-helix (46.04%) and irregular curl (48.34%), followed by extended chain (4.09%) and β-turn (1.53%).

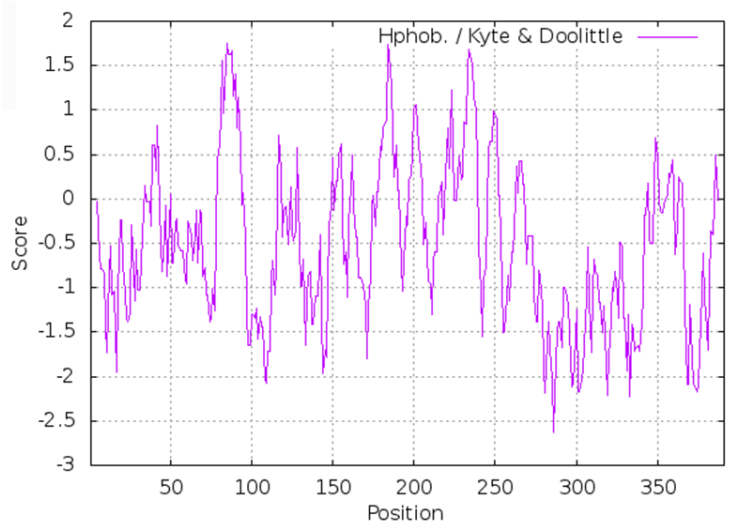

Figure 2 Hydrophobic, hydrophilic analysis of Gz*ABI5-3A3*

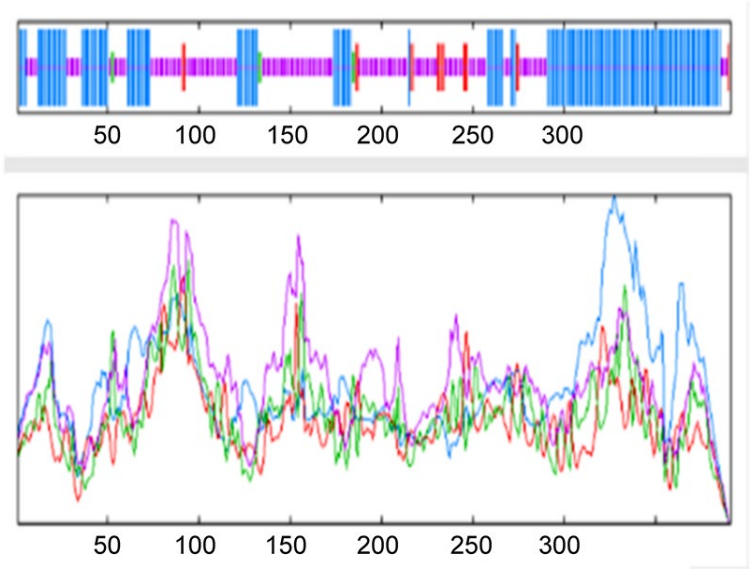

Figure 3 The predicated secondary structure of Gz*ABI5-3A3*

Note: Blue is α-helix; Purple is irregular curl; Red is extension chain; Green is β-turn

1.3 Cis-element of Gz*ABI5-3A3* gene promoter

In order to further understand the function of Gz*ABI5-3A3* gene, the cis-element of 1 891 bp of the upstream of Gz*ABI5-3A3* gene promoter was analyzed. It was found that it contains a variety of cis-elements. In addition to the core elements TATA box and CAAT box of the promoter itself, there are also a variety of cis-elements involved in various reactions (Table 1). ARE and GC-motif are cis-elements necessary for anaerobic induction; AuxRR-core and TGA-element are involved in auxin responsiveness; TATC-box is gibberellin-responsive element; ABRE is involved in the abscisic acid responsiveness; CGTCA-motif, TGACG-motif and TCA-element are involved in the MeJA-responsiveness and salicylic acid responsiveness; G-Box, TGGC-motif and Sp1 are light responsive elements; It also contains a MYB binding site and a MYB recognition site. It can be inferred that Gz*ABI5-3A3* may be involved in the regulation of the growth, photosynthesis, flowering, seed germination and anthocyanin accumulation of plant.

Table 1 Cis element analysis of Gz*ABI5-3A3* gene promote

Name of element	Source plant	Site	Signal sequence	Function
ABRE	*Arabidopsis thaliana*	172, 1 523	ACGTG	Cis-acting element involved in the abscisic acid responsiveness
ARE	*Zea mays* L.	241	AAACCA	Cis-acting regulatory element essential for the anaerobic induction
AuxRR-core	*Nicotiana tabacum* L.	604	GGTCCAT	Cis-acting regulatory element involved in auxin responsiveness
CGTCA-motif	*Hordeum vulgare* L.	155	CGTCA	Cis-acting regulatory element involved in the MeJA-responsiveness
G-Box	*Zea mays* L.	171, 685	CACGTT	Cis-acting regulatory element involved in light responsiveness
GC-motif	*Zea mays* L.	1 435, 1 605	CCCCCG	Enhancer-like element involved in anoxic specific inducibility
GTGGC-motif	*Hordeum vulgare* L.	1 484	CAGCGTGTGGC	Part of a light responsive element
P-box	*Oryza sativa* L.	1 333	CCTTTTG	Gibberellin-responsive element
RY-element	*Helianthus annuus* L.	1 269	CATGCATG	Cis-acting regulatory element involved in seed-specific regulation
Sp1	*Oryza sativa* L.	558, 904	GGGCGG	Light responsive element
TATC-box	*Oryza sativa* L.	230	TATCCCA	Cis-acting element involved in gibberellin-responsiveness
TCA-element	*Nicotiana tabacum* L.	1 722	CCATCTTTTT	Cis-acting element involved in salicylic acid responsiveness
TGA-element	*Brassica oleracea* L.	1 448, 1 539	AACGAC	Auxin-responsive element
TGACG-motif	*Hordeum vulgare* L.	155	TGACG	Cis-acting regulatory element involved in the MeJA-responsiveness
MYB recognition site	*Arabidopsis thaliana*	2 576	CCGTTG	-
Myb-binding site	*Nicotiana tabacum* L.	2 288	CAACAG	-

1.4 Amino acid homology of Gz*ABI5-3A3* gene

Phylogenetic tree analysis showed that Gz*ABI5-3A3* has the highest homology with Ta*ABI5D-SH-31*, Ta*ABI5D-SH-23* and Ta*ABI5D-SW-23* in *Triticum aestivum* (Figure 4). The results of sequence alignment between Gz*ABI5-3A3* and Ta*ABI5D-SH-31*, Ta*ABI5D-SH-23* as well as Ta*ABI5D-SW-23* showed that the similarity of these four genes was 87.78% (Figure 5).

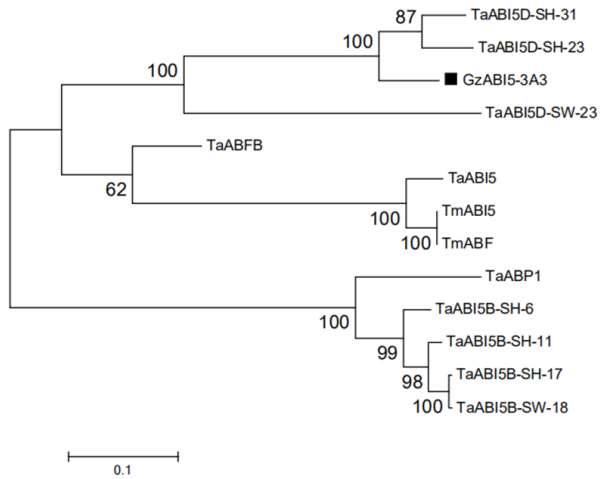

Figure 4 Phylogenetic tree of Gz*ABI5-3A3*

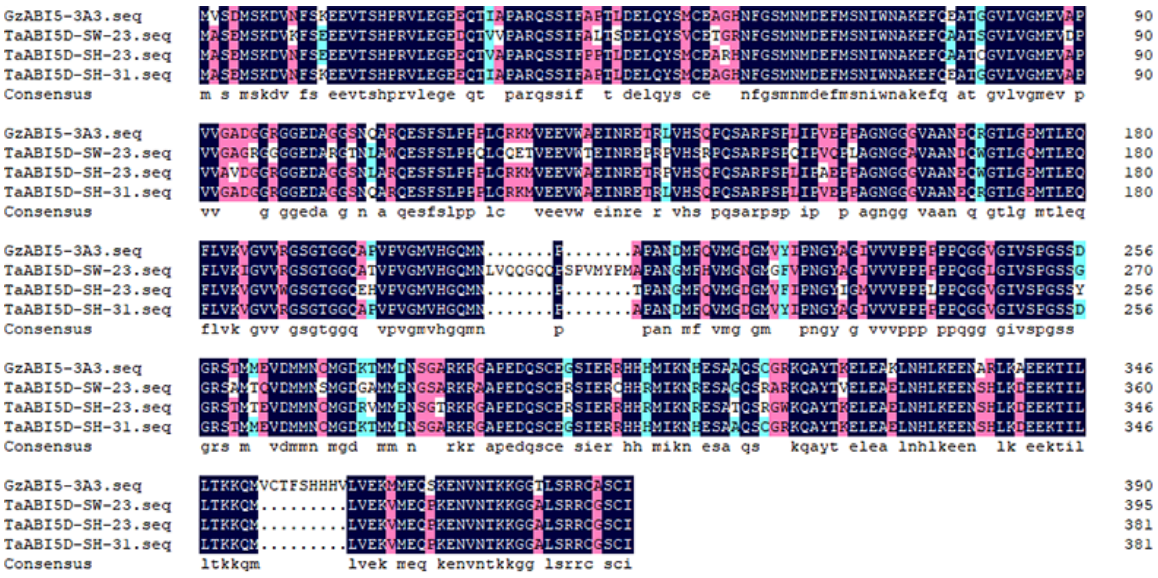

Figure 5 Multiple alignment of Gz*ABI5-3A3* with Ta*ABI5D-SW-23,* Ta*ABI5-SH-23* and Ta*ABI5-SH-31* in wheat

1.5 Acquisition and validation of Gz*ABI5-3A3* overexpressed transgenic tobacco plants

The leaf discs of sterile tobacco seedlings were placed in the co culture medium for dark culture for 3 days, and then the co cultured leaves were transferred to the screening differentiation medium for light culture. They were subcultured once in 7 days. After callus and 2 cm differentiation buds grew, the differentiation buds were cut and transferred to the rooting medium, and transplanted after adventitious roots grew. DNA extraction and PCR identification of transformed tobacco were carried out. Wild *Nicotiana benthamiana* and water were used as negative control and bacterial solution as positive control. PCR results showed that the transgenic plants could amplify bands with the same fragment size as the bacterial solution, while the negative control could not amplify bands (Figure 6), which proved that PBI121-Gz*ABI5-3A3* had been successfully transferred into tobacco, and a total of 8 transgenic tobacco strains were obtained.

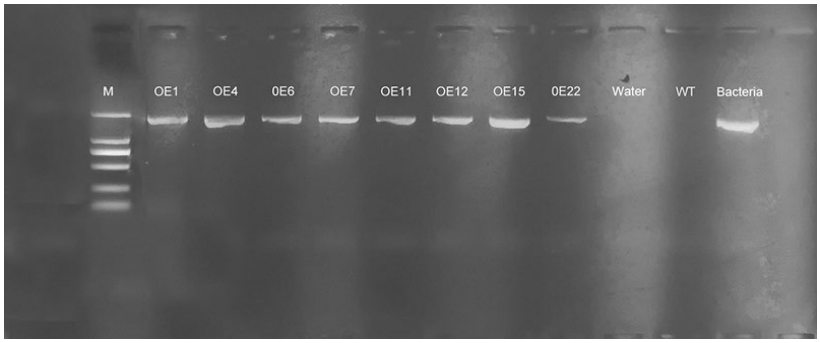

Figure 6 Validation of Gz*ABI5-3A3* transgenic plants

Note: M: DL 2000 Marker; OE1, OE4, OE6, OE7, OE11, OE12, OE15, OE22: Gz*ABI5-3A3* overexpressed positive plants; Water, WT: Negative control; Bacteria: Positive control

1.6 Anthocyanin content and gene expression of anthocyanin synthesis pathway in transgenic plants

The anthocyanin content of three overexpressed transgenic lines L4, L7 and L15 and the wild type were determined. The results showed that the anthocyanin content of transgenic plants L4, L7 and L15 were significantly lower than that of the wild type (Figure 7). Using overexpressing plants L4, L7 and L15 and wild tobacco cDNA as templates, the expression levels of Nt*PAL*, Nt*CHS*, Nt*DFR* and Nt*ANS* in tobacco leaves were analyzed by qRT-PCR. The results showed that the expression of Nt*PAL* in L4 was significantly lower than that in wild tobacco, and the expression of Nt*PAL* in L7 and L15 was extremely significantly lower than that in wild tobacco (Figure 8B); The expression levels of Nt*CHS*, Nt*DFR* and Nt*ANS* in L4, L7 and L15 were extremely significantly lower than those in wild tobacco (Figure 8A; Figure 8C; Figure 8D).

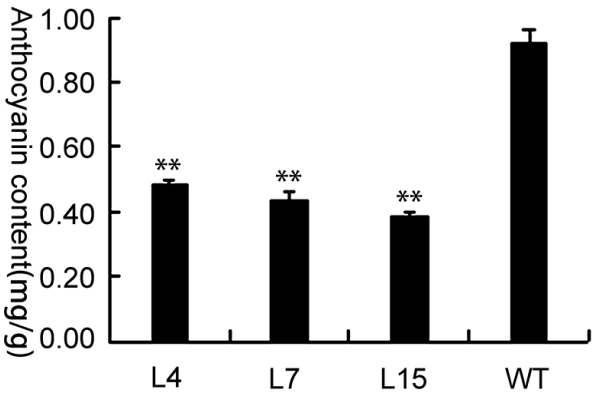

Figure 7 Anthocyanin content in leaves of wild-type tobacco and Gz*ABI5-3A3* overexpressed transgenic tobacco

Note: WT: Wild type; L4, L7, L15: Gz*ABI5-3A3* overexpressed transgenic plants; * means significant difference (p<0.05), ** means very significant difference (p<0.01)

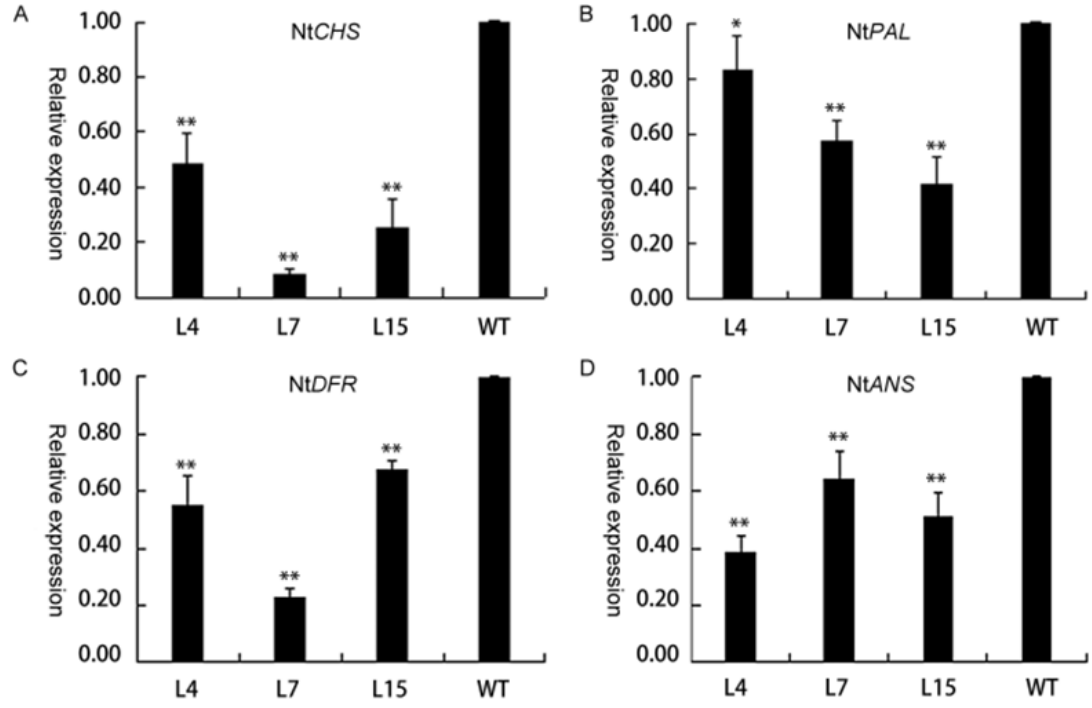

Figure 8 Wild-type tobacco and Gz*ABI5-3A3* overexpressed transgenic tobacco leaves

Note: A: Nt*CHS;* B: Nt*PAL;* C: Nt*DFR;* D: Nt*ANS*; WT: Wild type; L4, L7, L15: Gz*ABI5-3A3* overexpressed transgenic plants; * means significant difference (p<0.05), ** means very significant difference (p<0.01)

2 Discussion

In this study, the full-length cDNA of Gz*ABI5-3A3* was cloned from 'Guizimai 1'. Sequence analysis showed that Gz*ABI5-3A3* contained a bzip conserved domain, belonging to A subgroup of bzip family. Chang et al. (2019) showed that ABI5 is a kind of bzip transcription factor induced by ABA, which not only participates in the dormancy and germination of seeds (Sun et al., 2015; Zhao et al., 2016), regulates the growth of plants (Cheng et al., 2014; Li et al., 2019; Qi et al., 2020), but also regulates the accumulation of anthocyanins (Hoth et al., 2010; Cao and Liu, 2019). An et al. (2017) showed that in apple (*Malus pumila* Mill.), the bzip transcription factor Md*HY5* regulates anthocyanin accumulation by regulating the expression of *MdMYB10* gene and downstream anthocyanin biosynthesis gene. Chen et al. (2020) showed that ABA signal induces anthocyanin accumulation in *Arabidopsis thaliana* seedlings and may regulate anthocyanin synthesis through the synergistic effect of *ABI5* and *MBW* complex. Through the cis-element analysis of Gz*ABI5-3A3* promoter, it was found that the Gz*ABI5-3A3*

promoter region contains MYB binding sites and recognition sites, which was consistent with the research results of Chen et al. (2020). It can be speculated that Gz*ABI5-3A3* may bind to MYB transcription factors and participate in the regulation of anthocyanin accumulation.

In order to further verify the function of Gz*ABI5-3A3* in anthocyanin synthesis, the gene was overexpressed in tobacco and 8 transgenic lines were obtained. Liu et al. (2018) found that the bzip transcription factor Sl*HY5* in tomato (*Lycopersicon esculentum* Miller) regulates anthocyanin biosynthesis by binding the G box or ACE motif site to the promoters of anthocyanin biosynthesis genes *CHS1*, *CHS2* and *DFR*. Hoth et al. (2010) found that ABI5 mutant can promote anthocyanin synthesis under 3% sucrose treatment, indicating that ABI5 can negatively regulate anthocyanin synthesis. In this study, three overexpressed lines L4, L7 and L15 were selected to determine their anthocyanin content. The results showed that the anthocyanin content of tobacco leaves of overexpressed lines was significantly lower than that of wild type, which was the same as that of Hoth et al. (2010). The gene expression of Nt*PAL*, Nt*CHS*, Nt*DFR* and Nt*ANS* in anthocyanin synthesis pathway in Gz*ABI5-3A3* overexpressed line was further studied. It was found that its expression was significantly lower than that of wild type. The results showed that Gz*ABI5-3A3* can negatively regulate anthocyanin synthesis by inhibiting the expression of structural genes related to anthocyanin synthesis pathway. However, the molecular mechanism of how Gz*ABI5-3A3* regulates anthocyanins remains to be further studied.

3 Materials and Methods

3.1 Test materials

The wheat variety 'Guizimai 1' with purple grain was selected and preserved by Guizhou Branch of National Wheat Improvement Center. It was approved by Guizhou Crop Variety Approval Committee in June 2015 with the approval number of Qianshenmai No. 2015003. The seeds of 'Guizimai 1' with full grains were planted in the Experimental Site of Guizhou Branch of National Wheat Improvement Center (26°25'N, 106°40'E). The ears with consistent flowering were selected for listing, marking, continuous observation and recording at the flowering stage, and the grains 25 days after flowering were selected as samples. The tobacco used for tobacco genetic transformation is *Nicotiana benthamiana* provided by our central laboratory.

3.2 Preparation of plant genomic DNA, RNA and cDNA

TaKaRa MiniBEST Plant RNA Extraction Kit was used to extract total RNA from grains of 'Guizimai 1' at different stages. The RNA concentration was detected by ultraviolet visible spectrophotometer (Genove Nano), 2 μL RNA were taken and the quality of RNA was detected by 1% agarose gel electrophoresis. The reverse transcription kit PrimeScript™ 1st Strand cDNA Synthesis Kit was used to reverse transcribe the total RNA into cDNA and the concentration was detected.

3.3 Construction of overexpression vector of Gz*ABI5-3A3*

Through the CDS sequence of Gz*ABI5-3A3*, the specific amplification primers Gz*ABI5-3A3*-F1 and Gz*ABI5-3A3*-R1 were designed (Table 2). The cDNA obtained by reverse transcription was used as the template for amplification. The amplified product was connected to the overexpression vector pBI121, transformed into the competent state of *Escherichia coli* Stbl2, coated on LB solid medium containing kanamycin, incubated in darkness at 37℃ for 12~16 hours, and the positive single colony was selected and sent to Sangon Biotech (Shanghai) Co., Ltd. for sequencing. After obtaining the correct clone, it was transformed into the competent state of *Agrobacterium* LBA4404 for tobacco genetic transformation.

3.4 Analysis of Gz*ABI5-3A3* gene sequence and coding protein

The primary structure, secondary structure and promoter cis-elements of Gz*ABI5-3A3* protein were analyzed by online software such as ProtParam, Plantcare, InterProScan and SOPMA. The homologous sequence of Gz*ABI5-3A3* was searched by BlastP and BlastX in NCBI. The phylogenetic tree was constructed by MEGA7 to determine the highly homologous sequences. The homologous sequences were compared by DNAMAN to preliminarily infer the function of Gz*ABI5-3A3*.

Table 2 primer sequences

Primer name	Primer sequence(5′-3′)	Purpose
Gz*ABI5-3A3*-1	F: CGCGGATCCATGGTGTCGGACATGAGCAAG	PCR amplification
	R: TCCGGGCCCTCACCAGATGCAGCTAGCGC	
35S	F: CCACGTCTTCAAAGCAAGTG	Positive plant validation
Gz*ABI5-3A3*-2	R: TCACCAGATGCAGCTAGCGC	
Nt*Actin*	F: AATGATCGGAATGGAAGCTG	Transgenic tobacco qRT-PCR
	R: TGGTACCACCACTGAGGACA	
Nt*PAL*	F: CAAGAACGGTGGTGCTCTTC	
	R: CCAGAACCAACTGCAGTACC	
Nt*CHS*	F: GTACAACTAGTGGTGTAGACA	
	R: CCAACTTCACGAAGGTGAC	
Nt*DFR*	F: AACCAACAGTCAGGGGAATG	
	R: TTGGGCATCGAGAGTTCCAG	
Nt*ANS*	F: TGGCGTTGAAGCTCATACTG	
	R: GGAATTAGGCACACACTTTG	

Note: The restriction sites are underlined

3.5 Genetic transformation of Gz*ABI5-3A3* tobacco

The young leaves were taken from the upper part of tobacco sterile seedlings, cut into 1 cm×1 cm cubes and pre-cultured in MS medium at 28℃ for 3 days, then the pre-cultured leaves were infected in heavy suspension for 6 minutes, and dark cultured in co culture medium for 3 days. Then the co cultured leaves were placed in screening differentiation medium for light culture, subcultured once in 7 days. After callus and 2 cm differentiation buds grew, the differentiation buds were cut and transferred to rooting medium, and transplanted after adventitious roots grew (Figure 9).

3.6 Identification of transgenic plants

DNAsecure Plant Kit (TIANGEN) was used to extract the DNA of transgenic plants. *Agrobacterium* bacterial solution was used as the positive control, and wild *Nicotiana benthamiana* and water were used as the negative control. The primer 35S-F was located on the vector, Gz*ABI5-3A3*-R2 was located on the target gene, and the amplification length was 1 521 bp. PCR reaction procedure was as follows: 95℃ for 5 min, 95℃ for 30 s, 60℃ for 30 s, 72℃ for 90 s, and for 30 cycles, 72℃: 5 min; Store at 4℃. 5 μL PCR products were taken and were detected by 1% agarose gel electrophoresis.

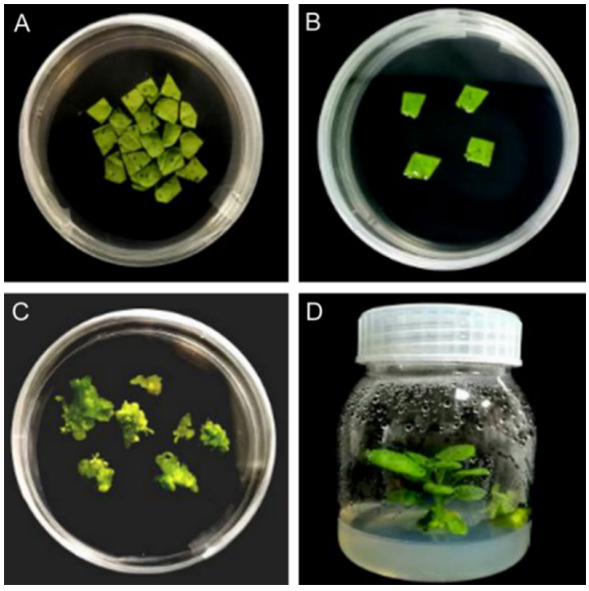

Figure 9 Transgenic plants of Gz*ABI5-3A3*

Note: A: Co culture; B: Screening differentiation; C: Callus and growth; D: Rooting culture of differentiation

3.7 Determination of anthocyanin content in transgenic plants

The extraction method of anthocyanins from tobacco leaves adopted the method of Pattanaik et al. (2010). Transgenic tobacco leaves grown in the medium for 1 month were used as materials.

3.8 Expression analysis of anthocyanin synthesis pathway genes in transgenic plants

The expression levels of Nt*PAL*, Nt*CHS*, Nt*DFR* and Nt*ANS* in transgenic tobacco leaves were detected by Real-time PCR. Among them, Nt*Actin* is an internal reference gene. The reaction system was as follows: 2 μL template cDNA, 1 μL upstream primers, 1 μL downstream primers, 12.5 μL SYBRII, and 8.5 μL ddH_2O. PCR reaction procedure was as follows: 95℃ for 30 s, 95℃ for 5 s, 51℃ for 30 s, for 40 cycles, and each sample repeated 3 times. The instrument used was Bio-Red CFX 96 Touch Real-time PCR, and the relative expression was calculated by $2^{-\Delta\Delta ct}$ (Livak and Schmittgen, 2001).

Authors' contributions

PYS was the experimental designer and executor of this research, completing data analysis and writing the first draft of the manuscript; DYB participated in experimental design and analysis of experimental results; RMJ, XRH and LLH were the conceivers and the persons in charge of the project, guiding the experimental design, data analysis, manuscript writing and modification. All authors read and approved the final manuscript.

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