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Original Research

Yeast One-Hybrid Screening for Regulators of *IbWD40* in Purple-Fleshed Sweet Potato (*Ipomoea batatas* [L] Lam.)

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Abstract

Background: The transcription regulator *IbWD40* is known to be involved in anthocyanin biosynthesis in purple-flesh sweet potato (*Ipomoea batatas*). However, little is known about the upstream transcription regulators on the promoter of *IbWD40*. **Methods**: Yeast one-hybrid screening was performed on the storage roots of purple-fleshed sweet potato to identity upstream transcription regulators on the promoter of *IbWD40*. Luciferase reporter assays and Yeast one-hybrid assays were used to verify these upstream binding proteins interacted with the promoter. Real-time PCR was used to analyze the gene expression of upstream transcription regulators, transcription factors, and structural genes involved in anthocyanin biosynthesis in different root stages of purple-fleshed and white-fleshed sweet potato. **Results**: IbERF1, IbERF10, IbEBF2, IbPDC, IbPGP19, IbUR5GT, IbDRM, IbPPA and IbERF73 were identified as candidate binding proteins for the promoter of *IbWD40*. Furthermore, IbERF1, IbERF10 and IbERF73 were identified as upstream transcription regulators on the promoter of *IbWD40* involved in anthocyanin biosynthesis. **Conclusions**: IbERF1, IbERF10 and IbERF73 were identified as transcription regulators on the promoter of *IbWD40*, which is involved in the regulation of anthocyanin biosynthesis in purple-fleshed sweet potato.

Keywords: anthocyanin biosynthesis; IbERF1; IbERF10; IbERF73; transcription regulators

1. Introduction

Anthocyanin have anticancer and antioxidation functions, and has been shown to lower blood pressure and prevent arteriosclerosis [1,2]. Moreover, anthocyanins have important biological significance for the adaptation of plants to their environment. These compounds can improve plant photoprotection ability, increase resistance to cold and drought, and confer antibacterial and antioxidant resistance to biotic and abiotic stresses [3]. Not only do they have important biological roles in plant defense and protection, anthocyanins also have major clinical impacts. These include nutritional and pharmacological effects, such as prevention of cardiovascular diseases, antitumor and antimutation properties, and the prevention and treatment of diabetes. Such properties are based on the antioxidant activity of anthocyanin [4]. The biosynthesis and regulation of anthocyanin is influenced by environmental and genetic factors that form a complex regulatory network [5,6]. Two types of gene control the biosynthesis of anthocyanin. One includes structural genes that encode catalytic enzymes in the anthocyanin synthesis pathway. The other type includes transcription factor genes encoding proteins that bind to the promoters of structural genes and regulate their transcription.

Transcription factors such as R2R3-MYBs, bHLH and WD40 have been demonstrated to regulate anthocyanin biosynthesis in plants [7]. The WD40 repeat motif mediates protein–protein interactions by acting as a fixative during these interactions. Yeast two-hybrid experiments have identified the MYB- and BHLH-type transcription factors as the main ligand proteins interacting with the WD40 domain. WD40 proteins are involved in a variety of physiological processes in plants, including abiotic stress, growth and development, and flavonoid synthesis. WD40 has also been reported to regulate anthocyanin biosynthesis in *Arabidopsis* [8], petunia [9], corn [10], purple perilla [11], duck wheat [12], alfalfa [13], grapevine [14], pomegranate [15], apple [16] and strawberry [17].

IbWD40 has been implicated in anthocyanin biosynthesis in purple-fleshed sweet potato [18]. However, the transcription regulators on the promoter of *IbWD40* (*PIbWD40*) involved in anthocyanin biosynthesis in the storage roots of purple-fleshed sweet potato have yet to be reported. In the present study we therefore used yeast one-hybrid screening to search for transcription regulators of *PIbWD40*. In plant, the ethylene response factor AP2/ERF involved in multiple physiological processes in plant growth [19,20]. IbERF1, IbERF10 and IbERF73 were identified here as transcription regulators of *PIbWD40* in the regulation of anthocyanin biosynthesis in purple-fleshed sweet potato.

2. Materials and Methods

2.1 Plant Materials

Purple-fleshed sweet potato *cv.* A5 and white-fleshed sweet potato *cv.* Yubeibai were cultivated in the biological garden at South China Normal University, Guangzhou, Guangdong, China. *Arabidopsis* (*Arabidopsis thaliana*) used for subcellular localization and for dual luciferase assays was cultivated in a growth chamber with a day/night cycle of 16/8 h at 20 ± 2 °C.

2.2 Extraction of Genomic DNA and RNA, Gene Isolation and Sequence Analysis

A small amount of root tissue (0.5 g) was ground to a fine powder in the presence of liquid nitrogen using a mortar and pestle. A plant genomic DNA kit (Cat. No. 4992201, Tiangen, Beijing, China) was used to extract DNA and a HiPure Plant RNA Midi kit (Cat. No. R4152, Magen, Guangzhou, China) was used to extract RNA. In order to eliminate the possibility of DNA contamination, total RNA was treated with DNAse I enzyme from an RNase-free kit (TaKaRa, Kyoto, Japan). DNA concentrations and purity were determined using a BioPhotometer plus (Eppendorf, Hamburg, Germany). cDNA synthesis was performed with oligo (dT) as primer using M-MLV reverse transcriptase according to the manufacturer's recommendations (cat. no. A5001, Promega, Wisconsin, USA). PCR products were judged on 1.2% agarose gels, ligated into plasmid and sequenced (Sangong, Shanghai, China).

2.3 Yeast One-Hybrid (Y1H) Screening

Y1H were performed using the Matchmaker Gold Yeast One-Hybrid Library Screening System according to a previous report [21]. Total RNA was isolated to construct the prey cDNA library (TaKaRa, Japan). A cDNA pool was inserted into pGADT7-Rec. Two amplifications promoter fragments *IbWD40-1* (–1136 bp~–585 bp) and *IbWD40-2* (–584 bp~–1 bp) were inserted into pAbAi. The pAbAi-bait plasmids were linearized and transformed into the yeast strain Y1HGold. Transformants were selected on plates containing a selective synthetic dextrose medium lacking uracil. The linear pGADT7-Rec vector was co-transformed into the bait yeast strains and selected on synthetic dextrose (SD)/-Leu/AbA plates. The primers used in the Y1H screening are listed in **Supplementary Table 1**.

2.4 Yeast One-Hybrid Assay (Y1H)

Y1H was performed using the Matchmaker Gold Yeast One-Hybrid System as described in a previous study [22]. This identified IbERF1, IbERF10, IbEBF2, IbPDC, IbPGP19, IbUR5GT, IbDRM, IbPPA and IbERF73 as interacting with the *IbWD40* promoter. The promoter for *IbWD40* was inserted into pAbAi. Complete CDSs for IbERF1, IbERF10, IbEBF2, IbPDC, IbPGP19, IbUR5GT, IbDRM, IbPPA and IbERF73 were separately inserted into the pGADT7, which were then transferred into the bait strain and grown on SD/-Leu/AbA plates. Primers used for Y1H are listed in **Supplementary Table 1**.

2.5 Yeast Two-Hybrid Assay (Y2H)

The transcriptional activity of IbERF1, IbERF10, IbEBF2, IbPDC, IbPGP19, IbUR5GT, IbDRM, IbPPA and IbERF73 was investigated using the Matchmaker™ Gold Yeast Two-Hybrid System (Clontech, Shanghai, China). Full-length coding sequences for *IbERF1*, *IbERF10*, *IbEBF2*, *IbPDC*, *IbPGP19*, *IbUR5GT*, *IbDRM*, *IbPPA* and *IbERF73* were cloned into the pGBKT7 vector to construct bait-BD vectors. The PGBKT7-bait and PGADT7-empty vectors were then co-transferred into the yeast strain Y2HGold and grown on SD/-Trp plates. Positive colonies were transferred and grown on SD/-His-AbA plus plates. Then, positive colonies were transferred and grown on SD/-His-AbA X-a-Gal plus plates to observe the growth and color of the yeast colonies. The interaction between pGADT7-53 and pGBKT7-53 was used as a positive control, and the interaction between pGADT7-53 and pGBKT7 was used as a negative control. The primers used for Y2H are listed in **Supplementary Table 1**.

2.6 Dual-Luciferase Assay

Dual-luciferase assays were used to measure the transactivation activities of IbERF1, IbERF10, IbEBF2, IbPDC, IbPGP19, IbUR5GT, IbDRM, IbPPA and IbERF73 on the *IbWD40* promoter. In brief, full-length cDNAs for *IbERF1*, *IbERF10*, *IbEBF2*, *IbPDC*, *IbPGP19*, *IbUR5GT*, *IbDRM*, *IbPPA* and *IbERF73* were inserted into the pGreen II 0029 62-SK vector, with *Sac I* and *Xho I*, and the *IbWD40* promoter was inserted into the pGreen II 0800-LUC vector with *Sac I* and *Xho I*. Both constructs were transformed into *Arabidopsis* protoplasts as described previously [23]. The ratio of enzyme activity for LUC and REN was measured with an E1910 Dual-Luciferase® Reporter Assay System (Promega, Wisconsin, USA). Three independent experiments were carried out to study each interaction between transcription factors and promoters. For each interaction between transcription factors and promoters, three independent experiments were carried out, with three replicates in each experiment. A luciferase gene from Renilla driven by a 35S promoter in the luciferase vector acted as the positive control. Mixtures containing each transcription factor and empty vector 62-SK were also tested on the promoter as a control. The primers used for the dual-luciferase assay are listed in **Supplementary Table 1**.

2.7 Subcellular Localization

To analyze subcellular localization, the CDSs for upstream transcription factors without the stop codon were amplified and cloned with *BamH* I and *Hind* II into the pCambia1300 vector containing the GFP gene and *UBQ* promoter. Both constructs were transformed into *Arabidop-*

sis protoplasts as described previously [23]. GFP fluorescence was observed using a Zeiss confocal microscope (LSM710). Primers used for the subcellular localization analysis are listed in **Supplementary Table 1**.

2.8 Real-Time Quantitative PCR

Real-time quantitative PCR was used to evaluate the expression of upstream transcription factors (*IbERF1*, *IbERF10*, *IbERF73*), transcription factors (*IbMYB1*, *IbbHLH2*, *IbWD40*) and structural genes (*IbCHI*, *IbCHS*, *IbF3H*, *IbF3'H*, *IbDFR*, *IbANS*, *IbUF3GT*) in the fibrous roots, thick roots and storage roots of purple-fleshed sweet potato *cv.* A5 and white-fleshed sweet potato *cv.* Yubeibai. RT–qPCR was then performed using SYBR® Premix Ex Taq™ II (TaKaRa, Kyoto, Japan) in a total 20 µL reaction volume containing 100 ng of template cDNA, 0.5 µM of each primer, and 10 µL of SYBR® Premix Ex Taq™ II. The Bio-Rad CFX96 Real-Time PCR system (BIO-RAD, California, USA) was used according to the manufacturers' instructions. The amplification program was as follows: one cycle of 95 °C for 10 sec, followed by 40 cycles of 95 °C for 5 sec and 60 °C for 30 sec. IbG14 was used as the internal control and the comparative Ct analysis method was performed. The primers used for RT-qPCR are listed in **Supplementary Table 1**.

2.9 Statistical Analysis

Three biological replicates of each sample were subjected to one-way analysis of variance (ANOVA). Significant differences were calculated using SPSS 21.0 Statistics (SPSS Inc., Chicago, IL, USA) and Tukey's honest test ($p < 0.05$). Figures were drawn with SigmaPlot 12.3 (Systat Software Inc., San Jose, CA, USA).

3. Results

3.1 Screening of Transcription Regulators for the IbWD40 Promoter

Two amplifications of *IbWD40-1/2* promoter fragments were ligated into pAbAi vector to generate the pAbAi-bait plasmid. The minimum inhibitory concentration of AbA for the bait strains was determined according to the system user manual. This was found to be 300 ng/mL for the pAbAi-*PIbWD40-1* strains. The AbA concentration was 900 ng/mL to inhibited the positive strains (pGADT7-53 + p53-AbAi). Self-activation of *PIbWD40-2* could not be suppressed because the pAbAi-*PIbWD40-2* strains cannot be inhibited.

A total of 453 colonies of *PIbWD40-1* screened positive, and 175 binding proteins for *PIbWD40-1* were identified by the Y1H assay. The gene sequences for *pIbWD40-1* are shown in **Supplementary Table 2**. The proteins IbERF1, IbERF10, IbEBF2, IbPDC, IbPGP19, IbUR5GT, IbDRM, IbPPA and IbERF73 were found to interact with the promoter for *IbWD40-1*.

3.2 Transcriptional Activity of the Transcription Regulators IbERF1, IbERF10, IbEBF2, IbPDC, IbPGP19, IbUR5GT, IbDRM, IbPPA and IbERF73

Y2H assays results showed that pGADT7-53 + pGBKT7-53 and pGBKT7-transcription regulators + pGADT7-empty transformed strains can grow on SD/-Trp, SD/-His-AbA plus, and SD/-His-AbA X-a-Gal plus plates, and the color of emerging yeast colony was blue (Fig. 1). These results indicate that IbERF1, IbERF10, IbEBF2 and IbERF73 had transcriptional activity.

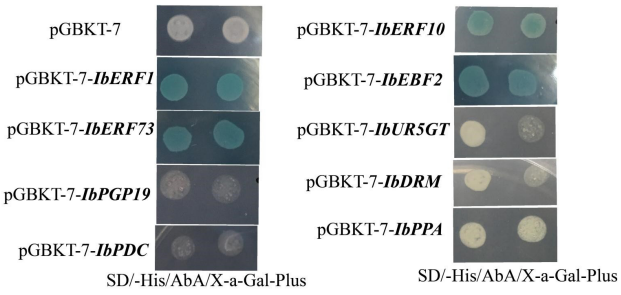

Fig. 1. Transcriptional activation of transcription regulators in yeast cells. Y2H Gold strains successfully transformed with corresponding vector and grown on the SD/-His/AbA/X-α-Gal plates at 30 °C for 3–5 days. The two points of each treatment were grown on a petri dish. Transcription activation was monitored by the growth status of yeast cells using the X-α-Gal assay.

3.3 Interaction between Transcription Regulators and PIbWD40-1

Y1H was performed to identify whether the transcription regulators identified above (IbERF1, IbERF10, IbEBF2, IbPDC, IbPGP19, IbUR5GT, IbDRM, IbPPA, IbERF73) interacted with *PIbWD40-1*. The assay results showed the positive control could grow on SD/-Leu/AbA plates, but not the negative control. The transformed strains grew on SD/-Leu/AbA plates (Fig. 2). These results demonstrate interaction between transcription regulators and *PIbWD40-1*.

Dual-luciferase assays were performed to confirm the interactions between transcription regulators and *PIbWD40-1*. IbERF1, IbERF10, IbEBF2, IbPDC, IbPGP19, IbDRM, IbPPA and IbERF73 all showed significant activation of *PIbWD40-1* (Fig. 3), thus confirming their interaction with *PIbWD40-1*.

3.4 Subcellular Localization of the Transcription Regulators

In the subcellular localization assays, the green fluorescence of the GFP control was observed in the nucleus and cytoplasm. Green fluorescence from *IbERF1/IbERF10/IbPDC/IbPGP19/IbPPA/IbERF73*-GFP fusions were detected only in the nucleus (Fig. 4), suggest-

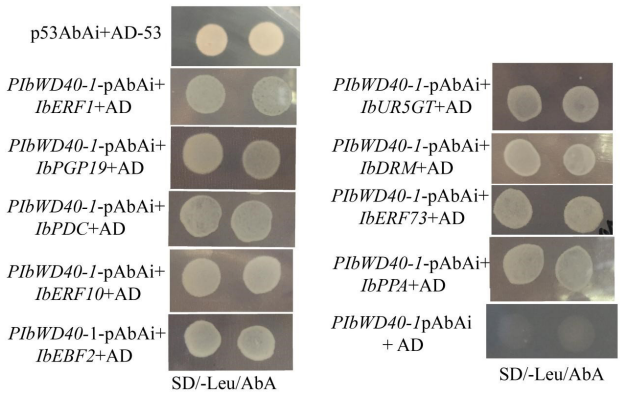

Fig. 2. Interaction between transcription regulators and *PIbWD40-1* studied using yeast one-hybrid assays. Y1H Gold strains successfully transformed with corresponding vector and grown on SD/-Leu/AbA plates at 30 °C for 3–5 days. The two points of each treatment were grown on a petri dish. Interaction was confirmed by the growth status of yeast cells.

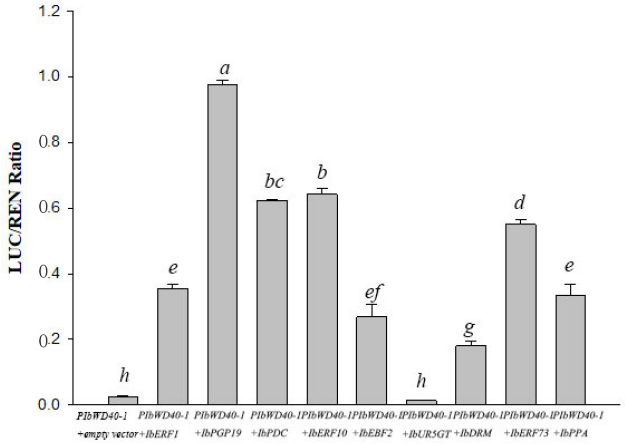

Fig. 3. The IbERF1, IbERF10, IbEBF2, IbPDC, IbPGP19, IbDRM, IbPPA and IbERF73 proteins activate *PIbWD40-1* in dual-luciferase assays. Error bars represent standard deviation (SD). The significance tests are shown as a, b, c, d, e, f, g, h. A different lowercase roman alphabet in the column chart indicates a significant difference ($p < 0.01$).

ing that IbERF1, IbERF10, IbPDC, IbPGP19, IbPPA and IbERF73 were nuclear proteins. The IbUR5GT, IbDRM and IbEBF2 proteins were expressed in both the nucleus and cytoplasm.

3.5 Expression Characteristics of Transcription Regulators

Real-time PCR was used to analyze the gene expression of transcription regulators, transcription factors and structural genes involved in anthocyanin biosynthesis at different root stages of purple- and white-fleshed sweet potato (Fig. 5). The expression levels of transcription factors and structural genes at different root stages of purple-fleshed

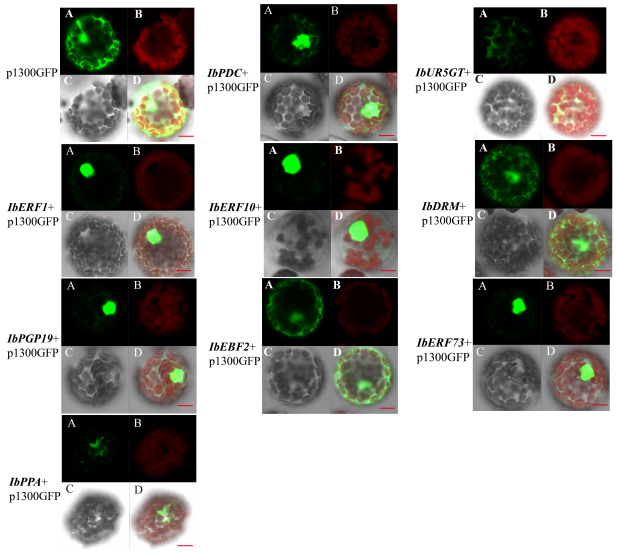

Fig. 4. Subcellular localization of transcription regulators in *Arabidopsis* protoplasts. The fusion protein and GFP control were transiently expressed in *Arabidopsis* protoplasts. Bars represent 20 μm. A: GFP; B: Chloroplast; C: Light field; D: Merged graph.

sweet potato were found to be higher than in white-fleshed sweet potato. However, the expression of *IbERF1* at different root stages was quarter in purple-fleshed sweet potato compare with in white-fleshed sweet potato, indicating negative regulation by *IbERF1* of anthocyanin accumulation. The expression of *IbERF10* and *IbERF73* at different root stages of purple- and white-fleshed sweet potato were not associated with anthocyanin accumulation, indicating the existence of more complex mechanisms in the regulation of anthocyanin biosynthesis.

4. Discussion

Anthocyanin biosynthesis in plants is regulated by the MBW complex. Other transcription factors including ERF, WRKY, COP1 and NAC proteins have also been implicated in the regulation of anthocyanin biosynthesis [24–27]. WRKY domain conserved 60 amino-acid that precisely prevails the interaction with W-boxes of targeted gene promoters [25]. The NAC family contains a conserved activation domain at the C-terminus and a DNA binding domain at the N-terminus [27]. In this study, Y1H screening was used to identify the transcription regulators of *PIbWD40* involved in anthocyanin biosynthesis. Our results revealed that IbERF1, IbERF10 and IbERF73 act as transcription regulators of *PIbWD40-1*, which in turn regulates anthocyanin biosynthesis.

In recent years, the interaction between MYB and bHLH proteins has been studied extensively, especially with regard to the regulation of anthocyanin synthesis. The transcription factor PdMYB118 directly interacts with the bHLH transcription factor PdTT8 to regulate injury-

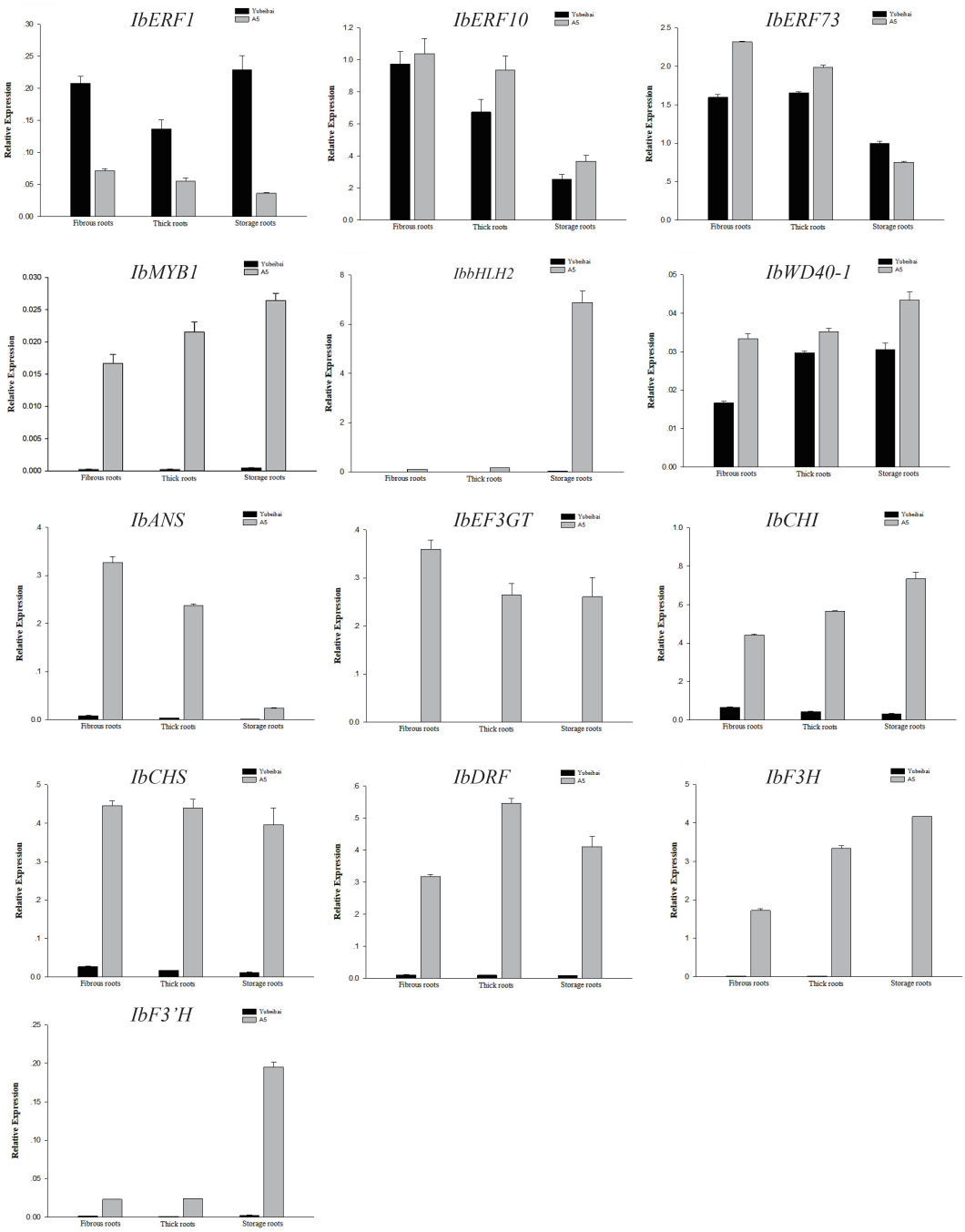

Fig. 5. Relative expression levels of transcription regulators, *IbWD40* and structural genes involved in anthocyanin biosynthesis at different root stages of purple-fleshed and white-fleshed sweet potato. Fibrous roots (diameter <2 mm), Thick roots (2 mm< diameter >5 mm), Storage roots (diameter >5 mm).

induced anthocyanin synthesis in aspen [28]. In plants, the transcription factor WD40 interacts with MYB and bHLH to regulate anthocyanin synthesis. MYB111 and WRKY40 are involved in the regulation of anthocyanin synthesis in red apple [29], while AWD40 interacts with DkMYB2 and DkMYB4 to regulate the synthesis of proanthocyanin in persimmon [30]. However, anthocyanin synthesis has different regulatory mechanisms in the red pear. Co-expression of MYB10 and bHLH33 or single expression of WD40 lead to different pear skin colors [31]. In laurel, the MRWD40-1 protein interacts with MYB and bHLH to enhance the accumulation of anthocyanin [32]. It has also been reported that WRKY is involved in the regulation of anthocyanin synthesis, with WRKY40 being implicated in the apple. The flavonoid synthesis pathway is thus highly organized, with key structural genes and a large number of transcription factors being involved in the process.

The ethylene response factors AP2/ERF are a family of transcription factors involvedin plant growth [33]. ERF transcription factors have been shown to regulate the biosynthesis of anthocyanins in plants. In carrot cells grown in suspension, DcERF1 can upregulate *DcPAL3* promoter activity, and DcERF2 also has involved in anthocyanin biosynthesis [34]. AP2/ERF transcription factors regulate anthocyanin biosynthesis in Salvia miltiorrhiza flowers, where significantly different expression levels were observed between purple and white flowers in transcriptome data [35]. ERF transcription factors have also been shown to regulate anthocyanin biosynthesis in various species. Seven PsERFs were positively correlated with PsMYB10 involved in anthocyanin biosynthesis in plum [36]. In *Arabidopsis*, the double mutants *aterf4* and *aterf8* reduced anthocyanin accumulation, thus indicating positive regulation of anthocyanin biosynthesis by AtERF4 and AtERF8 [37]. ERF transcription factors are also involved in light-induced anthocyanin biosynthesis in pears [38]. In conclusion, the present study found that IbERF1, IbERF10 and IbERF73 interact with the promotor of *IbWD40-1* to regulate anthocyanin biosynthesis in purple-fleshed sweet potato.

5. Conclusions

IbERF1, IbERF10 and IbERF73 were identified as transcription regulators on the promoter of *IbWD40*, which is involved in the regulation of anthocyanin biosynthesis in purple-fleshed sweet potato.

Availability of Data and Materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Author Contributions

FG designed the research. RL coordinated the study and interpreted the results. DF performed experiments, wrote the manuscript, analysed the results and conceived the study. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work to take public responsibility for appropriate portions of the content and agreed to be accountable for all aspects of the work in ensuring that questions related to its accuracy or integrity.

Ethics Approval and Consent to Participate

Purple-fleshed and white-fleshed sweet potato were used in this study. They were kindly provided by Pro. Bing (South china nromal university, Guanzhou, China).

Acknowledgment

Not applicable.

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Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/j.fbl2809200.

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