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Research article

A novel *glutathione S-transferase* gene from sweetpotato, *IbGSTF4*, is involved in anthocyanin sequestration

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ABSTRACT

Anthocyanins are synthesized by multi-enzyme complexes localized at the cytoplasmic surface of the endoplasmic reticulum (synthesis site), and transported to the destination site, the vacuole. Three mechanisms for the vacuolar accumulation of anthocyanin in plant species have been proposed. Previous studies have indicated that *glutathione S-transferase* (*GST*) genes from model and ornamental plants are involved in anthocyanin transportation. In the present study, an anthocyanin-related *GST*, *IbGSTF4*, was identified and characterized based on transcriptome results. Phylogenetic analysis revealed that IbGSTF4 was most closely correlated to PhAN9 and CkmGST3, the anthocyanin-related GST of *Petunia hybrida* and Cyclamen. Furthermore, the expression analysis revealed that *IbGSTF4* is strongly expressed in pigmented tissues, when compared to green organs, which is in agreement to the ability to correlate with anthocyanin accumulation. A GST activity assay uncovered that the IbGST4 protein owned similar activities with the GST family. Furthermore, the molecular functional complementation of *Arabidopsis thaliana* mutant *tt19* demonstrated that *IbGSTF4* might play a vital role in the vacuole sequestration of anthocyanin in sweetpotato. Moreover, the dual luciferase assay revealed that the LUC driven by the promoter of *IbGSTF4* could not be directly activated by *IbMYB1*, suggesting that the regulatory mechanism of anthocyanin accumulation and sequestration in sweetpotato was intricate.

1. Introduction

Sweetpotato (*Ipomoea batatas* L.) is an important food crop in China, and ranks fifth after rice, wheat, maize and cassava in developing countries (Fu et al., 2014). Purple-fleshed sweetpotato (PFSP) has been known to process high contents of anthocyanin in its storage roots, which is considered as a functional food for improving human health (Lim et al., 2013).

In plants, anthocyanin is a class of flavonoid pigments, which is not only responsible for the red, purple and blue colors of plant tissues, but also plays an important role in different physiological processes, such as pollination, seed distribution, etc. (Harborne and Williams, 2000). Anthocyanins are initially synthesized in the cytosol, and transported to vacuoles, where it is sequestrated to exhibit color formation in different plant organs (Zhao and Dixon, 2010). Previous researches have mainly focused on enzyme-coding structural genes and transcription factor genes involved in sweetpotato anthocyanin biosynthesis (Mano et al., 2007; Zhou et al., 2010; Guo et al., 2015). Meanwhile, gene encoding proteins that take part in anthocyanin vacuolar sequestration in sweetpotato have not been understood at present. To date, only three types of mechanisms have been presumed to be involved in anthocyanin- and flavonoid-transport, including glutathione S-transferase (GST), vesicle trafficking and membrane transporters (Zhao, 2015). However, the question on how these distinct but nonexclusive mechanisms work together for anthocyanin compartmentalization remains to be addressed.

GSTs are a superfamily that consists of multifunctional enzymes that catalyze the conjugation of the tripeptide glutathione (GSH, γ-

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glutamylcysteinyl glycine) to electrophilic sites. Based on amino acid sequence homology and gene structure conservation, seven soluble GSTs and one microsomal GST were subdivided (Mohsenzadeh et al., 2011). Moreover, soluble GSTs were classified into phi (GSTF), tau (GSTU), theta (GSTT), zeta (GSTZ), lamda (GSTL), dehydroascorbate reductase (DHAR) and tetrachlorohydroquinone dehalogenase (TCHQD). GSTs have been found to be involved in various abiotic and biotic stress resistance and plant development (Piero et al., 2006; Piero, 2015). Recently, several anthocyanin-related *GST* genes have been identified in different plant species, including *Zea mays* (Marrs et al., 1995), *Petunia hybrida* (Alfenito et al., 1998), *Arabidopsis thaliana* (Kitamura et al., 2004), *Perilla frutescens* (Yamazaki et al., 2008), *Vitis vinifera* (Conn et al., 2008; Pérez-Díaz et al., 2016), Cyclamen (Kitamura et al., 2012), *Litchi chinensis* (Hu et al., 2016), and strawberry (Luo et al., 2018). Therefore, the present study aims to study the importance of GSTs in anthocyanin accumulation through the characterization and identification of *IbGSTF4*, which encodes the GSTF of sweetpotato.

2. Materials and methods

2.1. Plant materials and samples collection

Five sweetpotato (*Ipomoea batatas* L.) cultivars, including 'Xuzi 3' (dark-purple fleshed cultivar, DC), 'Xuzi 1' (light-purple fleshed cultivar, LC), 'Xu 32' (yellow-fleshed cultivar, YC), 'Xuyu 34' (red-fleshed cultivar, RC) and Xu 28 (white-fleshed cultivar, WC), were selected and planted in the Sweetpotato Research Institute, Xuzhou, China. Samples of young leaves, mature leaves, stems, fibrous roots, pencil roots and storage roots of 'Xuzi 3' were collected. Each treatment was performed in three replicates, and all samples were immediately frozen in liquid nitrogen and stored at −80 °C for further use.

Wild-type Columbia, mutant *tt19-1* and transgenic *tt19-1* plants of *Arabidopsis thaliana* were used as model plants to study the function of the sweetpotato target gene. They were grown in a mixture of vermiculite, perlite and peat moss (1:1:1) in a growth chamber at 22 °C with a 16-h light and 8-h dark photoperiod.

2.2. Extraction of DNA and RNA and cDNA synthesis

Genomic DNA was isolated from young leaves of 'Xuzi 3' using the modified cetyltrimethylammonium bromide (CTAB) method (Kim and Hamada, 2005). Total RNAs were extracted using a plant RNA isolation Kit (Huayueyang Biotechnology Co., LTD., China), and treated with DNase I (Takara, Japan), according to manufacturer's protocols. Then, RNA integrity was assessed by electrophoresis on a denaturing agarose gel, and RNA quality and quantity were determined using a NanoDrop spectrophotometer (Thermo Fisher Scientific Inc., USA). First-strand cDNA was synthesized using the ReverTra Ace qPCR RT Master Mix with a gDNA Remover kit (Toyobo, Japan), according to manufacturer's instructions.

2.3. Sequence analysis and identification of IbGSTF4 in sweetpotato

Depending on the transcriptome analysis results (unpublished data), obtained using the illumina GAIIx system (ABlife. Inc., Wuhan, China) and Hiseq 2000 system (BGI. Inc., Shenzhen, China), a novel unigene, which is expected to be correlated with the anthocyanin pathway, was chosen for further work following three important parameters: (1) this unigene was presumably described similar to *AtTT19* (At3g03190), (2) the transcripts per kilobase million (TPM) values of purple-flesh sweetpotato varieties were higher than those of non-purple-flesh sweetpotatoes, and (3) the fold change of DT/WT and LT/WT were ≥2 at the same time. This unigene, comp93922_c0 (1232bp, called *IbGSTF4*), contains a complete coding sequence. The full-length cDNA and genomic DNA were extracted from the storage root of Xuzi 3, and the *IbGSTF4* gene was amplified using the following primers: IbGSTF4-F, 5′-ATGGTAGTTAAGGTGTTCGGTTCT-3′ and IbGSTF4-R, 5′-TCAATTTTTGTGGTTCATGAGGTCC-3'. The resulting PCR product was gel purified using an AxyPrep DNA Gel extraction Kit (Axygene, USA), cloned into the pEASY-Blunt Simple Cloning Vector, according to manufacturer's protocols (TransGen Biotech, China), and sequenced.

2.4. Bioinformatics analysis

The functionally conserved domains were predicted online (http://www.ncbi.nlm.nih.gov/structure/cdd/cdd.shtml). The structural analysis of the deduced protein was performed using ExPASy (http://www.expasy.org/tools/protparam.html). The protein sequences were aligned using ClustalX 1.83 (http://www.ebi.ac.uk), and a phylogenetic tree was constructed using the neighbor-joining method through the MEGA 5.0 software with the bootstrap method (1000 bootstrap replicates).

2.5. Gene expression analysis

Gene transcript levels were measured by quantitative RT-PCR using the One-Step Real-Time PCR System (Applied Biosystems, USA) with the SYBR Green Real-time PCR Master Mix (Toyobo, Japan), according to manufacturer's instructions. The qRT-PCR program was as follows: 1 min at 95 °C for denaturation, 40 cycles for 15 s at 95 °C, 15 s at 60 °C, and 20 s at 72 °C for amplification. The primers used for the qPCR analysis were as follows: IbGSTF4q-F, 5′-CGGCTATTGAGGACGGAGAC-3′ and IbGSTF4q-R, 5′-CATTGATCCACCACGGCTCT-3'. *IbARF* (ADP-ribosylation factor) was used as an internal control (Park et al., 2012). The comparative threshold cycle (Ct) method ($2^{-\Delta\Delta Ct}$ method) was used to calculate the relative expression level of the target genes (Livak and Schmittgen, 2001). All reactions were carried out in triplicate with three biological replicates.

2.6. Total anthocyanin analysis of sweetpotato

The total anthocyanin content of sweetpotato was determined according to the method described by Guo et al. (2015). The pigments of different plant materials were extracted with acidic methanol (1% HCl, w/v), and the absorbance at 530 nm and 657 nm were metrically in the photo using a UV-2450 Spectrophotometer (Shimadzu, Japan). Anthocyanin content was calculated using the following equation, which was performed by Guo et al. (2015): $Q_{Anthocyanin} = (A530–0.25A657) \times 0.1\ M^{-1}$. Each sample was replicated three times.

2.7. Functional expression of IbGSTF4 in Escherichia coli (E. coli) and GST activity assay

Using the pEASY-Blunt vector plasmid as a template, primers pET28a-IbGSTF4-F (*NcoI*), 5′-CATG ccatgg TAGTTAAGGTGTTCGGTTCT-3′ and pET28a-IbGSTF4-R (*XhoI*), 5′-CATG ctcgag ATTTTTGTGGTTCATGAGGTCCAACA-3′ were designed to amplify the cDNA sequence of *IbGSTF4*. The PCR products were purified and digested with *NcoI* and *XhoI*, and ligated to the pET28a (+) vector. The recombinant plasmid was transformed into *E. coli* TOP10 cells. Then, a single colony was inoculated and cultured at 37 °C in LB liquid medium containing 50 μg/mL of kanamycin with shaking at 200 rpm, until the optical density at 600 nm (OD 600) reached 0.6. In order to express *IbGSTF4* as a soluble protein, the final expression conditions were induced by the addition of isopropyl-β-Dthiogalactopyranoside (IPTG) to a final concentration of 0.5 mM at 37 °C for 2 h. Then, 13% SDS-PAGE electrophoresis was performed for the protein separation and detection. Cells of the sample (containing plasmid pET28a[+]-IbGSTF4) were harvested after 15 h of continuous induction at 15 °C, and sonicated. The recombinant IbGSTF4 protein was purified with using the 6XHis-tag Protein Purification Kit (ProbeGene, China), following the manual. The collected supernatant was used to measure GST activity, following the

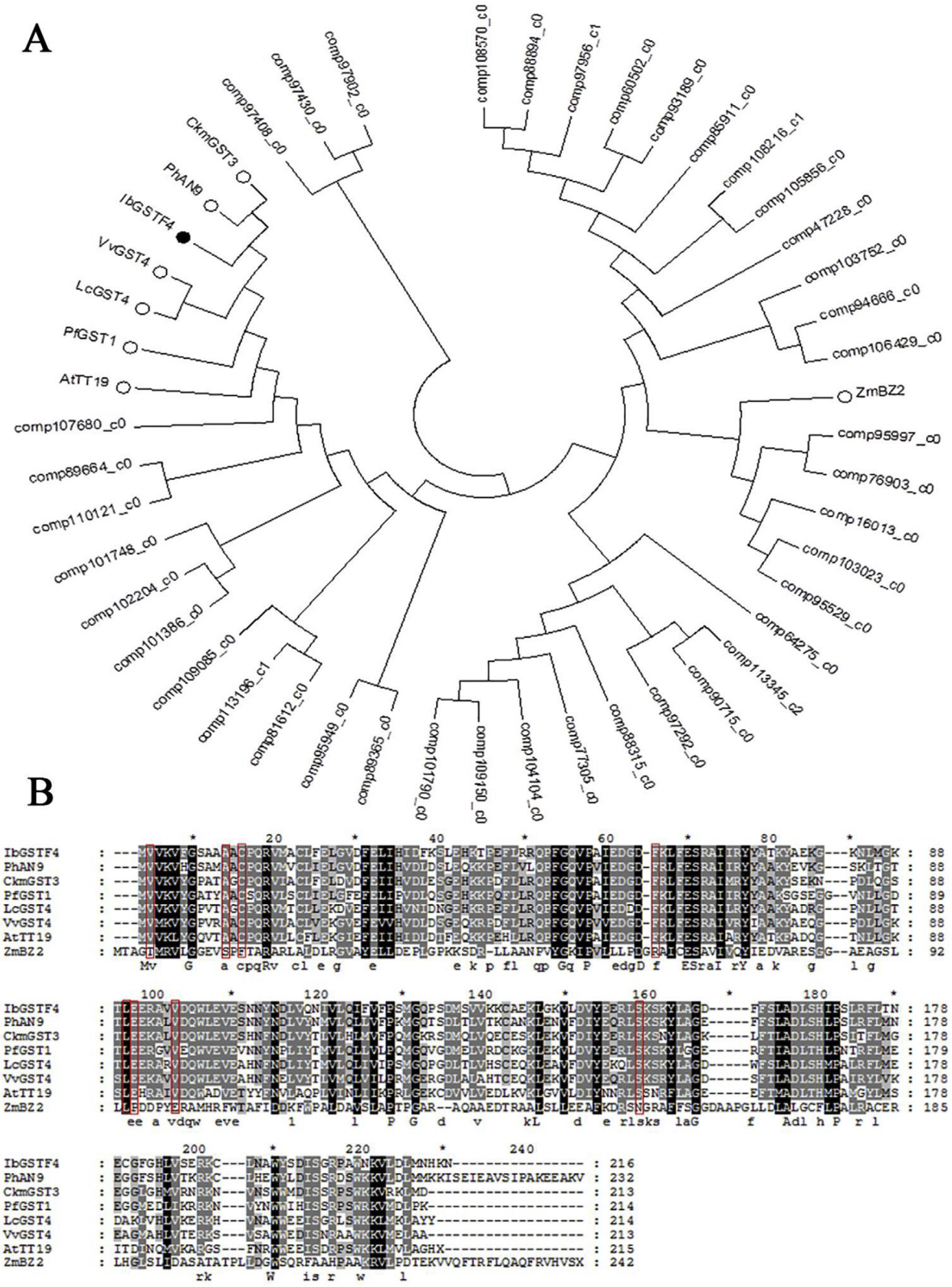

Fig. 1. Phylogenetic tree and protein sequence alignment of GSTs from sweetpotato and other plant species. (A) Phylogenetic comparison of IbGSTF4 and those anthocyanin-related GSTs. ZmBZ2 (*Zea mays*, AAA50245), AtTT19 (*Arabidopsis thaliana*, BAD89984), PhAN9 (*Petunia hybrida*, CAA68993), CkmGST3 (Cyclamen, BAM14584), VvGST4 (*Vitis vinifera*, AAX81329), PfGST1 (*Perilla frutescens*, BAG14300), and LcGST4 (*Litchi chinensis*, ALY05893). (B) Protein sequence alignment of IbGSTF4 and other plants. The numbers in the alignments indicate the amino acid positions. The red boxes show that the amino acid which are conserved in anthocyanin-related GST from di-cotyledons, according to Kitamura et al. (2012) and Hu et al. (2016). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

modification method described by Alfenito et al. (1998). Then, 0.1 mL of the supernatant was added to the final 3-mL reaction system, including 2.7 mL of reaction buffer (0.1 M of KH_2PO_4 and 1 mM of EDTA·2Na, pH = 6.5), 0.1 mL of 30 mM GSH, and 0.1 mL of 30 mM 1-Chloro-2,4-dinitrobenzene (CDNB). Absorbance at 340 nm was recorded using a UV-2450 Spectrophotometer (Shimadzu, Japan).

2.8. Expression vector construction and complementation analysis in the Arabidopsis mutant

After adding the base of A, the cDNA fragment was cloned into the Gateway pCR8/GW/TOPO vector using a TOPO TA Cloning kit (Invitrogen, USA), and the entry vector (pCR8/GW/TOPO:*IbGSTF4*) was constructed. Afterwards, pGWB12-*IbGSTF4* (containing cauliflower mosaic virus 35S [CaMV35S] promoter) was constructed after LR action using the Gateway LR Clonase Enzyme Mix (Invitrogen, USA). The final expression vector was introduced into *Agrobacterium tumefaciens* strain EHA105, and used in *Arabidopsis tt19-1* transformation through the floral dip method (Clough and Bent, 1998). The transgenic plants were grown on 1/2 MS medium plates containing 1.5% sucrose and 50 mg/L of kanamycin. The RT-PCR analysis of *IbGSTF4* was performed, and the *AtActin2* gene (At3g18780) was used as an internal reference. T_2 transgenic lines, which were grown on 1/2 MS medium containing 3% sucrose, were used for phenotypic investigations with respect to anthocyanin accumulation at the seedling stage and seed color. The stems of T_2 seedlings and *tt19-1* seedlings (control) were used for anthocyanin extraction determination, as described by Kitamura et al. (2012).

2.9. Molecular cloning and promoter analysis of IbGSTF4

Depending on the coding sequence of *IbGSTF4*, blast analysis was carried out using a recently published sweetpotato genome database (Yang et al., 2017). The core sites of the promoter region were predicted on line using the NDGP software (http://www.fruitfly.org/seq_tools/promoter.html). The promoter sequence of *IbGSTF4* was isolated from the leaves of 'Xuzi 3' using a set of primers: IbGSTF4pro-F, 5′-TCGCTGCATTAGTAACATGAACCTC-3′ and IbGSTF4pro-R, 5′-TGTTGGTGGATTATGGGTGTGTG-3'. The PCR product was cloned into a pMD19-T vector (TaKaRa, Dalian), and sequenced. Key cis-acting elements were analyzed using the PlantCARE program (http://www.bioinformatics.psb.ugent.be/webtools/plantcare/html/).

2.10. Dual luciferase assay

The promoter of the *IbGSTF4* gene was obtained using specific primers: IbGSTF4pro-F (*PstI*), 5′-CAG ctgcag TCGCTGCATTAGTAACATGAACCTC-3′ and IbGSTF4pro-R (*XbaI*), 5′-CAG tctaga TGTTGGTGGATTATGGGTGTGTG-3'. The PCR product was fused to the firefly luciferase (*Luc*) gene and the fusion construct pLL00R-*IbGSTF4pro-Luc* was generated (Zhang et al., 2015). The effector constructs of pCambia2300-*35S-IbMYB1a* was described by Kim et al. (2010). Both the pLL00R-*IbGSTF4pro-Luc* and pCambia2300-*35S-IbMYB1a* constructs were transformed into *Agrobacterium tumefacians* strain GV3101. The transient expression in the agroinfiltrated leaves of *Nicotiana benthamiana* (*N. benthamiana*) and subsequent dual luciferase assay was performed, as described by Zhang et al. (2015). Fluorescent signals were monitored *in vivo* using the imaging system (Tanon-5500, China).

3. Results

3.1. Isolation and characterization of IbGSTF4

Based on the previous transcriptome sequencing results of the investigators, 41 unigenes annotated as GST were acquired. In order to confirm the anthocyanin-related GSTs in sweetpotato, a phylogenetic tree was generated using the amino acid sequences of 41 IbGSTs and published anthocyanin-related GSTs (Fig. 1A).

The phylogenetic analysis of the deduced amino acids sequence of GST-like proteins (Fig. 1) indicated that IbGSTF4 (Accession number: MG873448) was most closely correlated to anthocyanin-related GST from di-cotyledon plants, such as petunia and cyclamen. At the same time, it was distinct from the maize BZ2 protein as a mono-cotyledon plant. In addition, the IbGSTF4 of *Ipomoea batatas*, VvGST4 of *Vitis vinifera*, PhAN9 of *Petunia hybrida*, CkmGST3 of Cyclamen, PfGST1 of *Perilla frutescens*, LcGST4 of *Litchi chinensis*, and AtTT19 of *Arabidopsis thaliana* all contain specific amino acids correlated to the anthocyanin pathway, and all have high homology among each other (marked by red boxes, Fig. 1B). On the other hand, these high-homology sites of anthocyanin-related amino acids were not conserved in ZmBZ2 (Fig. 1B).

The full-length cDNA sequence of *IbGSTF4* was amplified from the storage root of 'Xuzi 3', which was 651 bp. The deduced protein of IbGSTF4 contained 216 amino acids, with a molecular weight of 24.65 kDa and a theoretical isoelectric point of 6.53 (http://web.expasy.org/protparam/). The amino acids sequence of IbGSTF4 displayed the typical structure of the plant GST family with conserved GSH-binding C-terminal domain (GST_C) and N-terminal domain (GST_N) (http://blast.ncbi.nlm.nih.gov/; Fig. 2A). The *IbGSTF4* genomic DNA was also isolated, including 1339 nucleotides with two introns and three exons (Fig. 2B), which belong to phi-type GST proteins on the basis of amino acid similarity.

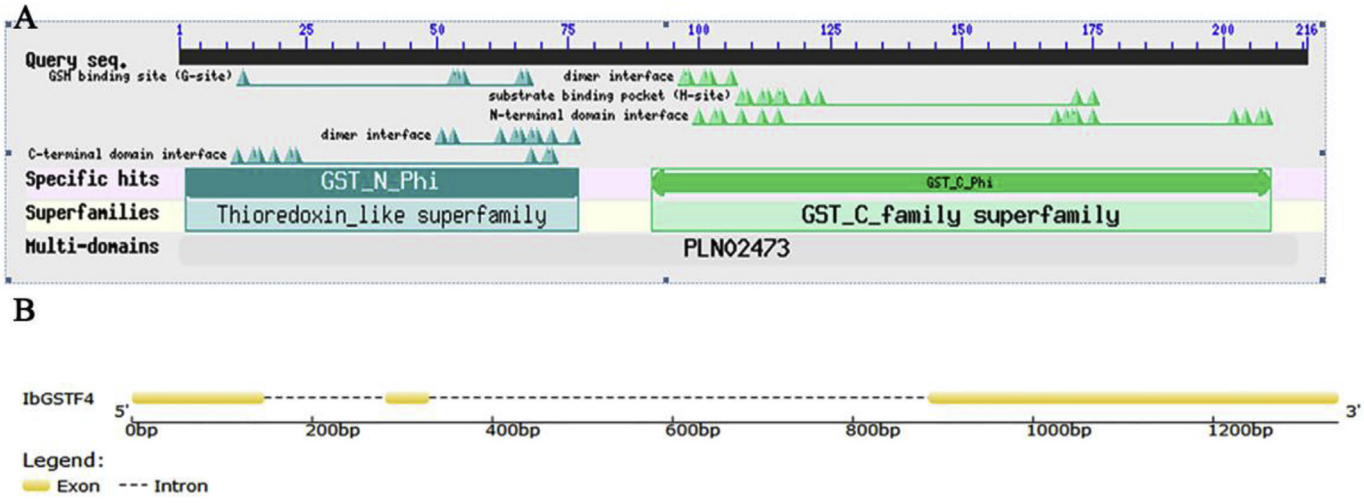

Fig. 2. Conserved domains and intron analysis of the *IbGSTF4* gene.

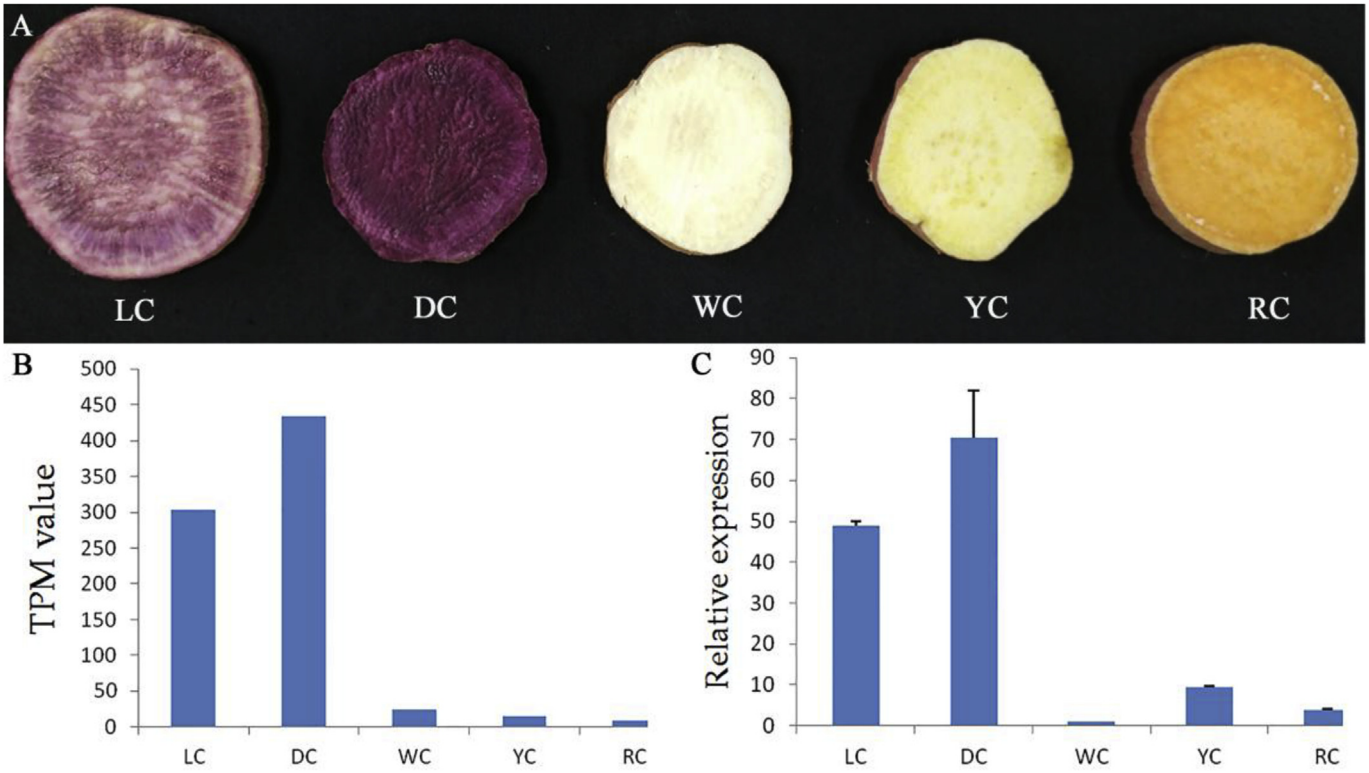

Fig. 3. Expression profiling of *IbGSTF4* in various sweetpotatos. An image of the storage root flesh color of different cultivars (A). The expression patterns of *IbGSTF4* were analyzed through the TPM values (B) and qRT-PCR (C). DC, dark-purple fleshed cultivar; LC, light-purple fleshed cultivar, YC, yellow fleshed cultivar; RC, red fleshed cultivar; WC, white fleshed cultivar. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

3.2. Expression profiling of IbGSTF4 related to anthocyanin accumulation in various cultivars and different tissues

The qRT-PCR analysis of *IbGSTF4* revealed that the expression levels in the storage roots of (light and dark) purple-fleshed sweetpotato varieties were approximately 50-fold higher than those of red, yellow and white-fleshed sweetpotoes, which is consistent with the RNA-seq (Fig. 3). From the above mentioned conclusions, the novel *IbGSTF4* gene (Accession number: MG873448) was discovered to be potentially correlated with anthocyanin sequestration in sweetpotato.

In the different organs of 'Xuzi 3', anthocyanin mainly accumulated in the pigmented parts, such as young leaf, stem, fibrous root, pencil root and storage root (Fig. 4A and B). The quantitative RT-PCR analyses were performed in accordance with the anthocyanin accumulation profiles. Predominant expression was detected in the storage root, followed by the pencil root and fibrous root. However, a very weak expression was observed in the green mature leaf and leaf petiole (Fig. 4C). These results suggest that *IbGSTF4* was involved in the anthocyanin biosynthesis of sweetpotato. From the above mentioned conclusions, the novel *IbGSTF4* gene was discovered to be potentially correlated with anthocyanin sequestration in sweetpotato.

3.3. IbGST4 shows GST activity in vitro

After the IPTG-induced expression of *IbGST4*, SDS-PAGE and Coomassie-blue staining indicated that the recombinant IbGST4 was successfully highly expressed. Its molecular mass was approximately 26 KDa, which was in accordance with the deduced sized of the IbGST4 protein (Fig. 5A). In order to determine whether IbGST4 was a GST, its ability to catalyze the GSH conjugate to CDNB was tested at an absorbance of 340 nm. Crude extracts from recombinant pET28a (+)-IbGSTF4 had GST activity, as indicated by its increase with the increase in measurement time, while the control group did not exhibit any GST activity (Fig. 5B). This quantitative test revealed that the *IbGSTF4* gene could be expressed in *E. coli*, and the expressed recombinant protein possesses the characteristic enzymatic activity of the GST family.

3.4. Complementation of the Arabidopsis tt19-1 mutant phenotype with the IbGSTF4 gene

A molecular functional complementation test was carried out to determine whether *IbGSTF4* could function in the regulation of anthocyanin accumulation in plants. The *IbGSTF4* cDNA driven by the CaMV35S promoter was transferred into the *tt19-1* mutant using *Agrobacterium*-mediated transformation. Transgenic seedlings harboring *IbGSTF4* were obtained, and expression analyses were performed (Fig. 6A). Under high-sucrose stress conditions, the accumulation of anthocyanin in hypocotyls was observed in transgenic *tt19-1* plants containing *IbGSTF4* at the seedling stage, and this was the same in wild-type *Arabidopsis*, but not in the mutant *tt19-1* (Fig. 6B and C). However, the seed color of the transformed plants after ripening was not recovered by visual inspection (data not shown). Therefore, *IbGSTF4* was correlated to the anthocyanin accumulation, but had different functions during *Arabidopsis* seed coat pigmentation.

3.5. Identification of the IbGSTF4 promoter and its interaction with IbMYB1

IbMYB1 is a key regulator of anthocyanin biosynthesis in sweetpotato (Park et al., 2015). Transgenic *IbMYB1a* Arabidopsis exhibits elevated levels of anthocyanin (Chu et al., 2013). The 2284 bp-length promoter region of the *IbGSTF4* gene was isolated from the genomic DNA of 'Xuzi 3' using the genome sequence (see the supplemental data).

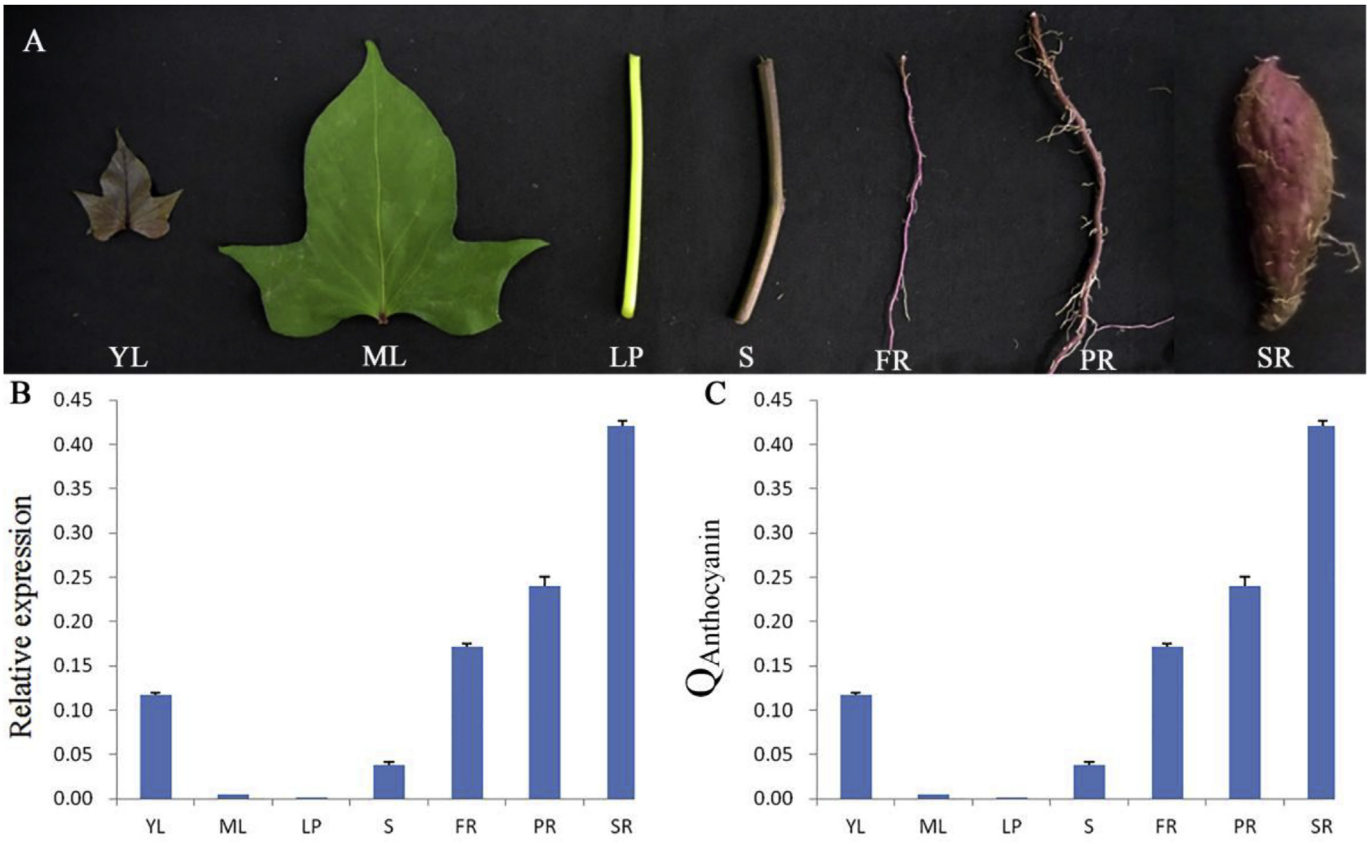

Fig. 4. Expression patterns of *IbGSTF4* in different tissues of 'Xuzi 3'. (A) Images of young leaves (YL), mature leaves (ML), leaf petiole (LP), stem (S), fibrous root (FR), pencil root (PR) and storage root (SR). (B) Anthocyanin content in different tissues. (C) The qRT-PCR analysis of *IbGSTF4* in different tissues. Data were presented as mean ± standard deviation (SD) in three independent replicates.

The *cis*-acting regulatory elements were analyzed using the PlantCARE database. Several important putative cis-regulatory elements, such as CAAT-box and TATA-box, and five MYB binding sites, including two MBSs and three MREs, were found in the promoter region correlated to drought and light responsiveness, respectively (Table 1). Moreover, other cis-acting elements involved in hormone, defense, stress and circadian responsiveness were also discovered. These results reveal that the expression of *IbGSTF4* might be controlled by various environmental factors.

IbMYB1 is a key regulator of anthocyanin biosynthesis in sweetpotato (Park et al., 2015). Transgenic *IbMYB1a* Arabidopsis exhibits elevated levels of anthocyanin (Chu et al., 2013). The reporter vector *IbGSTF4pro-luc* containing the *IbGSTF4* promoter mixed with the overexpression vector containing *IbMYB1* was used to determine the

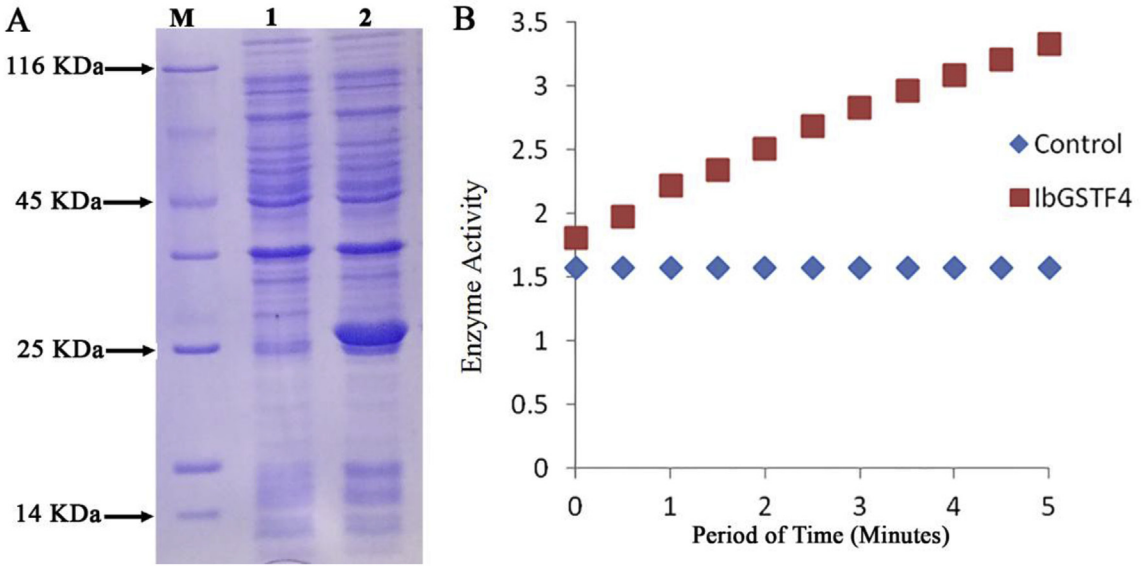

Fig. 5. GST activity assay. (A) SDS-PAGE analysis of recombinant IbGST4 under IPTG induction; M, protein Marker; Lane 1 and Lane 2, before and after IPTG induction, respectively. (B) Assay of IbGST4 enzyme activity *in vitro*.

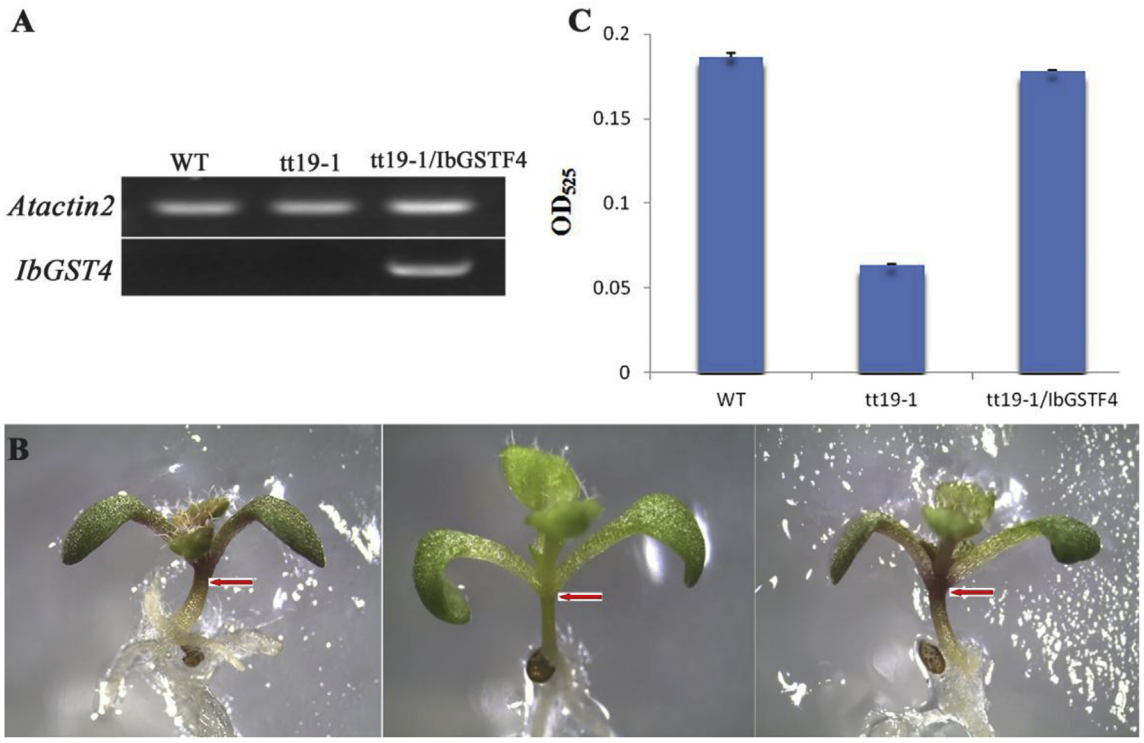

Fig. 6. Complementation of the *tt19-1* mutant with the *IbGSTF4* gene. Left to right: Wild-type Columbia (Col), *tt19*, and transgenic *tt19* seedlings harboring *IbGSTF4*. (A) Expression analysis of *IbGSTF4* in the *tt19-1*/35S-*IbGSTF4* transgenic lines. (B) Transgenic seedling of *IbGSTF4* exhibits anthocyanin accumulation (red arrow). (C) The quantification of anthocyanin in transgenic plants. Data were presented as mean ± standard deviation (SD) in three independent experiments. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 1
Responsive MYB binding sites of *IbGSTF4* promoter.

Site Name	Organism	Position	Strand	Matrix score.	sequence	function
MBS	*Arabidopsis thaliana*	151	–	6	CAACTG	MYB binding site involved in drought-inducibility
MBS	*Arabidopsis thaliana*	1099	+	6	CAACTG	MYB binding site involved in drought-inducibility
MRE	*Petroselinum crispum*	1390	–	7	AACCTAA	MYB binding site involved in light responsiveness
MRE	*Petroselinum crispum*	1483	–	7	AACCTAA	MYB binding site involved in light responsiveness
MRE	*Petroselinum crispum*	1576	–	7	AACCTAA	MYB binding site involved in light responsiveness

interaction between the *IbGSTF4* promoter and *IbMYB1*. The image did not reveal any signal in the part of the *N. benthamiana* leaf infiltrated with *IbGSTF4pro-luc* and 35S-*IbMYB1* together, while a signal could be captured in the positive control part (Fig. 7). The luciferase assay result revealed that *IbMYB1* could not directly activate the expression of *IbGSTF4*.

4. Discussion

Next-generation RNA-sequencing (RNA-seq) is a highly efficient and conventional method for gene discovery, especially in non-model plant species without reference genome sequences (Grabherr et al., 2011). In recent years, this technology has also been applied to the transcriptome analyses of color variations in some plants, such as yam (Wu et al., 2015), lettuce (Zhang et al., 2017), plum (González et al., 2016) and grape (Shangguan et al., 2017). At the same time, it may provide powerful tool to identify differentially expressed genes and uncover molecular basis (Ma et al., 2016). In the present study, RNA-seq results were used to discover a novel gene that participates in color formation in purple-fleshed sweetpotato storage roots.

In agreement with these present results, a previous study illustrated that a total of 42 full-length GST genes were discovered from two local transcriptome databases (DB12 and DB77 datasets) by Sanger sequencing (Ding et al., 2017). These 42 IbGSTs were divided into seven groups, including 27 Tau members, two Phi members, two Theta members, three Zeta members, three EF1Bγ members, three Lambda members, and two DHAR members (Ding et al., 2017). In the present study, in addition to the 40 unigenes, a novel Phi member (*IbGSTF4*, called after *IbGSTF1-3*, as previously referred by Ding et al. [2017]) was isolated from 'Xuzi 3', which was responsible for vacuolar anthocyanin sequestration. To date, a variety of *GST* genes have been identified from many plant species, including *Oryza sativa* (Soranzo et al., 2004) and *Arabidopsis thaliana* (Sappl et al., 2009). In addition, IbGSTF4 shared amino acid residues that were specific to anthocyanin-related GSTs from di-cotyledons (Kitamura et al., 2012). However, these high-homology sites were not conserved in *Zea mays* (marked by red boxes, Fig. 1B). These findings suggest that anthocyanin-related GSTs were derived from a single ancestral gene before the speciation of di-cotyledons and mono-cotyledons.

The expression patterns of anthocyanin biosynthesis-related genes in different tissues of sweetpotato have been tested (Zhou et al., 2010; Guo et al., 2015). In the present study, it was found that the expression of the *IbGSTF4* gene was higher in the pigmented storage root, pencil root, fibrous root, young leaves and stem, but lower in green mature leaves and leaf petiole, agreeing with the anthocyanin accumulation profiles in various organs (Fig. 4). A GST protein, LcGST4, presumably

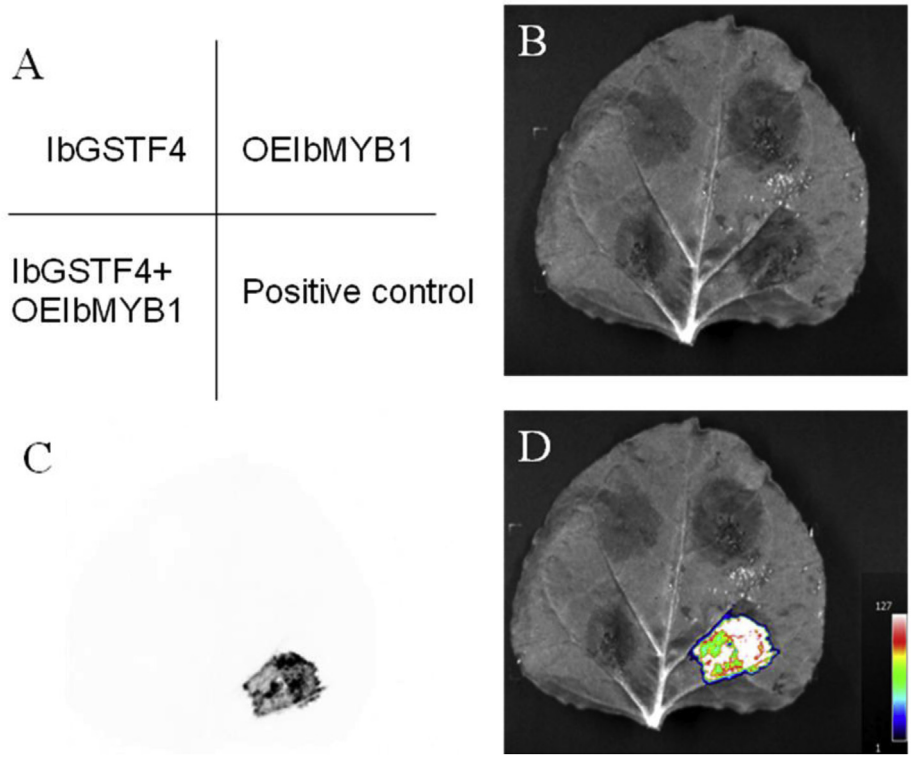

Fig. 7. Dual luciferase assay. A representative image of a *Nicotiana benthamiana* (*N. benthamiana*) leaf after infiltration with *IbGSTF4pro-luc* and 35S-*IbMYB1* is shown. (A) Experimental treatment arrangement diagram. (B) Image of a leaf under natural light. (C) Detection of fluorescence signal. (D) Image of the leaf after integration.

took part in anthocyanin accumulation in litchi fruit, and was previously reported to be highly expressed in pigmented tissues (Hu et al., 2016). From the results mentioned above, it was found that expression profiles of *IbGSTF4* were closely correlated with anthocyanin accumulation, suggesting the involvement of *IbGSTF4* in anthocyanin accumulation in sweetpotato.

In order to determine whether the purified IbGSTF4 protein was a GST, CDNB was used as a model substrate, in which most GSTs catalyze the conversion of this compound to dinitrophenol-glutathione (Marrs, 1996). Previous reports have indicated that maize BZ2 and petunia AN9 have the ability to conjugate GSH to CDNB *in vitro* (Marrs et al., 1995; Alfenito et al., 1998). Interestingly, GST activity could also be measured by supplying CDNB as a substrate using the crude extracts of injected *N. benthamiana* leaves containing the LcGST4 protein construct (Hu et al., 2016). In the present study, IbGSTF4 exhibited GST activity against the universal substrate CDNB at an absorbance of 340 nm.

Molecular complementation assays were used to test the function of *GST*-like genes in plants using the mutants in the present study. A carnation anthocyanin mutant *fl3* functionally complemented petunia *AN9* and maize *BZ2*, and was involved in the vacuolar sequestration of anthocyanin (Larsen et al., 2003). The *Arabidopsis tt19*, which is a knockout mutant of anthocyanin-related GST that displayed an anthocyanin-less phenotype, was complemented by the expression of petunia *AN9* (Alfenito et al., 1998), cyclamen *CkmGST3* (Kitamura et al., 2012), litchi *LcGST4* (Hu et al., 2016), and grapevine *VviGST1*, *VviGST3*, and *VviGST4* (Pérez-Díaz et al., 2016). Recently, a *Reduced Anthocyanins in Petioles* (*RAP*) gene encoded a GST anthocyanin transporter that was essential for the foliage and fruit coloration in strawberry (Luo et al., 2018). *RAP* could complement *tt19*-7, suggesting that *RAP* is the ortholog of *TT19* in *Arabidopsis* (Luo et al., 2018). In the present study, different expression levels of *IbGSTF4* in the *tt19* mutant could form the purple pigmentation of hypocotyls and cotyledons, but these failed to induce the color of the seed coats. These results indicate that the *IbGSTF4* gene was involved in the anthocyanin accumulation, but was not related in the pro-anthocyanidin pathway, which was not fully in accordance with *tt19* (Kitamura et al., 2004).

In general, anthocyanin biosynthesis is regulated by three transcriptional factors: R2R3-MYB protein, a basic helix-loop-helix (bHLH, MYC) protein, and WD40-type co-regulators (WD40). MYB transcription factors (TFs) play a key role in modulating structural genes that encode enzymes of the anthocyanin biosynthetic pathway (Allan et al., 2008). To date, a number of MYB TFs have been well-characterized in high plants (Borevitz et al., 2000). The overexpression of *PAP1* in transgenic *Arabidopsis* induced the upregulation of many anthocyanin-related genes, such as *phenylalanine ammonia-lyase* (*PAL*), *chalcone synthase* (*CHS*) and *dihydroflavonol 4-reductase* (*DFR*) (Borevitz et al., 2000). In addition, grape *MybA* genes control the anthocyanin biosynthesis *via* the expression of UDP-glucose: *flavonoid 3-O-glucosyltransferase* (*UFGT*) gene (Kobayashi et al., 2002). In sweetpotato, the R2R3-type *IbMYB1* was isolated from the purple-fleshed variety, which induced purple pigmentation in the flesh of storage roots (Mano et al., 2007). The overexpression of *IbMYB1* increased the transcription of structural genes related to anthocyanin biosynthesis, and generated dual-pigmented transgenic plants with high levels of both anthocyanins and carotenoids in storage roots (Park et al., 2015). However, molecular biological evidence has indicated that the regulation of anthocyanin-related *GSTs* with *MYB* genes was rare. In a previous study, Hu et al. reported that the expression of *LcGST4* was activated by *LcMYB1* (Hu et al., 2016). In contrast, the dual luciferase assay in the present experiment indicated that the expression of *IbGSTF4* was not directly regulated by *IbMYB1*, although several MYB binding sites were uncovered in the promoter upstream region. These results suggest that other *MYB* members may function in controlling the anthocyanin biosynthesis, or *IbMYB1* alone may not control the expression of genes for anthocyanin sequestration, and that it may function together with other regulatory factors (Supplemental Figs. 1–6). These imply that the regulatory mechanism of anthocyanin accumulation in sweetpotato is complicated. Therefore, more work should be conducted through further research.

Conflicts of interest

The authors declare no conflict of interest.

Author contributions

D.F.M. and Z.Y.L. designed the study, M.K., W.T., H.Y., and Z.X.S. conducted the experiments, Y.J.L. analyzed the results. M.K. and X.G.C. performed the figures and M.K. drafted the manuscript. M.H.A., Q.L. and D.F.M. revised the manuscript. All authors read and approved the final manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2018.12.028.

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