Cloning and Expression Analysis of IbGL3 in Sweet Potato

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Abstract

Purple-fleshed sweet potatoes are rich in anthocyanins and possess high edible and medicinal value. Anthocyanin biosynthesis is controlled by structural genes and regulatory genes. bHLH (basic helix-loop-helix protein) transcription factors can regulate the expression of multiple anthocyanin structural genes and play an important regulatory role in the anthocyanin biosynthetic pathway; however, there are currently no relevant reports on bHLH regulation of anthocyanin biosynthesis in sweet potato. In this study, based on sweet potato transcriptome data, a 2,120 bp bHLH gene IbGL3 was cloned from sweet potato using RT-PCR technology. This gene contains a 1,878 bp open reading frame, encoding 625 amino acids, with a protein molecular weight of 69.08 kDa and a theoretical isoelectric point (pI) of 5.20. The IbGL3 protein shares high homology with flavonoid synthesis-related bHLH proteins from other plants, all containing a conserved MIR region, bHLH domain, and ACT-like domain. Phylogenetic tree analysis revealed that IbGL3 clusters together with flavonoid-related bHLH proteins from other plants, belonging to subgroup f members. Expression analysis showed that the IbGL3 gene exhibited the highest expression level in deep purple sweet potatoes, followed by light purple sweet potatoes, and the lowest expression level in white-fleshed sweet potatoes, which is positively correlated with anthocyanin accumulation, suggesting that it plays an important regulatory role in the anthocyanin biosynthetic pathway of sweet potato. These findings contribute to further understanding of the function and mechanism of IbGL3 in sweet potato anthocyanin biosynthesis, laying a foundation for genetic improvement of anthocyanin biosynthesis in sweet potato.

Full Text

Cloning and Expression Analysis of IbGL3 in Ipomoea batatas

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Abstract

Purple-fleshed sweet potatoes are rich in anthocyanins and possess high nutritional and medicinal value. Anthocyanin biosynthesis is controlled by both structural genes and regulatory genes. The bHLH (basic helix-loop-helix) transcription factor plays a crucial regulatory role in anthocyanin biosynthesis by modulating the expression of multiple structural genes, yet no studies have reported bHLH-mediated regulation of anthocyanin biosynthesis in sweet potato. In this study, we cloned a bHLH gene named *IbGL3* from sweet potato (*Ipomoea batatas*) based on transcriptome data using RT-PCR technology. The full-length cDNA of *IbGL3* was 2,120 bp, containing a 1,878 bp open reading frame that encodes 625 amino acids. The encoded protein has a molecular weight of 69.08 kDa and a theoretical isoelectric point (pI) of 5.20. The instability index of 43.06 classifies IbGL3 as an unstable protein, while its grand average of hydropathicity of -0.555 indicates it is hydrophilic. Subcellular localization prediction revealed that IbGL3 is most likely localized in the nucleus, with a nuclear localization signal (PSSKKRKASKT) located at the C-terminus. Signal peptide analysis showed that IbGL3 lacks a signal peptide and is not a secretory protein, and transmembrane domain analysis confirmed it has no transmembrane domains. The IbGL3 protein shares high homology with bHLH proteins involved in flavonoid synthesis from other plants, containing conserved MIR, bHLH, and ACT-like domains. Phylogenetic analysis demonstrated that IbGL3 clusters with other plant flavonoid-related bHLH proteins, belonging to the IIIf subgroup. Expression analysis revealed that *IbGL3* expression was highest in deep purple-fleshed sweet potato, moderate in light purple-fleshed varieties, and lowest in white-fleshed sweet potato, showing a positive correlation with anthocyanin accumulation. These results suggest that IbGL3 plays an important regulatory role in sweet potato anthocyanin biosynthesis. This

study provides a foundation for further understanding the function and molecular mechanism of IbGL3 in anthocyanin biosynthesis and lays the groundwork for genetic improvement of anthocyanin content in sweet potato.

Keywords: *Ipomoea batatas*, anthocyanin, bHLH transcription factor, gene expression

Introduction

Anthocyanins are flavonoid compounds present in plants that play decisive roles in flower pigmentation, UV protection, and defense against pathogenic microbial invasion, while also offering significant nutritional and medicinal benefits (Panche et al, 2016; Lila, 2008). The anthocyanin biosynthetic pathway has been extensively studied in model plants, with relevant genes isolated from numerous species (Hichri et al, 2011; Albert et al, 2014; Wang et al, 2015). Plant anthocyanin biosynthesis is primarily controlled by two gene categories: structural genes encoding various enzymes involved in flavonoid biosynthesis, and regulatory genes encoding transcription factors that modulate flavonoid secondary metabolism by controlling multiple structural genes (Hichri et al, 2011; Wang et al, 2015). Research has confirmed that anthocyanin biosynthesis is mainly regulated by the MYB-bHLH-WD40 (MBW) transcriptional complex composed of MYB, bHLH, and WD40 transcription factors (Xu et al, 2015). The bHLH transcription factors contain a basic helix-loop-helix domain and can be divided into 12 subgroups (I-XII), with those regulating flavonoid biosynthesis concentrated primarily in the IIIf subgroup (Xu et al, 2015; Heim et al, 2003). In *Arabidopsis*, the main bHLH transcription factors regulating anthocyanin biosynthesis are TT8/AtbHLH42, GL3/AtbHLH1, and EGL3/AtbHLH2 (Gonzalez et al, 2008). The first bHLH gene regulating anthocyanin biosynthesis was isolated and identified in maize (Chandler et al, 1989), and since then, similar transcription factors have been isolated from various plants including chrysanthemum (*Chrysanthemum* spp.), apple (*Malus domestica*), and lychee (*Litchi chinensis*), most of which can activate the expression of multiple key enzyme genes in flavonoid biosynthesis (Xiang et al, 2015; Xu et al, 2017; Lai et al, 2016).

Sweet potato is a widely cultivated food and economic crop worldwide (Hu et al, 2016). As a health food recommended by the United Nations Food and Agriculture Organization, sweet potato is rich in starch, cellulose, and various beneficial secondary metabolites such as -carotene and anthocyanins (Liu et al, 2017). Among different sweet potato varieties, purple-fleshed sweet potatoes are particularly rich in anthocyanins with high edible and medicinal value (Li et al, 2013). However, research on anthocyanin biosynthesis and regulatory mechanisms in sweet potato lags behind that in model plants, and no reports have documented bHLH protein-mediated regulation of anthocyanin biosynthesis in this species (Li et al, 2014). In our previous study, comparative transcriptome

analysis of sweet potato varieties with different flesh colors identified a batch of differentially expressed genes associated with anthocyanin accumulation, including one EST sequence highly homologous to GL3 from other plants. This study aims to clone the *IbGL3* gene, perform bioinformatic analysis, and examine its expression patterns in relation to anthocyanin accumulation, thereby establishing a foundation for further elucidating its function and mechanism in anthocyanin biosynthesis.

Materials and Methods

Plant Materials

Sweet potato materials were planted at the Yongfa Base of Hainan Academy of Agricultural Sciences. Fresh storage roots from white-fleshed, light purple-fleshed, and purple-fleshed sweet potato varieties with consistent growth after five months were selected as experimental materials for RNA extraction and anthocyanin determination.

Anthocyanin Extraction and Quantification

Anthocyanins were extracted from sweet potato storage roots using 1% hydrochloric acid solution as the extraction solvent, and anthocyanin content was determined by spectrophotometry following the method described by Liu et al (2007).

Nucleic Acid Extraction and cDNA Synthesis

Total RNA was extracted from sweet potato storage roots using the Tiangen polysaccharide-polyphenol plant total RNA extraction kit. First-strand cDNA was synthesized from RNA using the Thermo Scientific First Strand cDNA Synthesis Kit according to the manufacturer' s instructions.

Gene Cloning and Sequence Analysis

Based on the EST sequence annotated as GL3 from transcriptome data, specific primers were designed: PGL3F (5 -GCCCAGTTTGTTCAAGAGCC-3) and PGL3R (5 -AGTTAGGGATAAACCTTTGCT-3). These primers were used to amplify *IbGL3* from sweet potato cDNA template. The amplification product was identified by agarose gel electrophoresis, the target band was recovered and ligated into the pMD19-T vector, and the construct was transformed into *E. coli* DH5 . Positive clones were selected through blue-white screening and colony PCR, then sequenced by Nuosai Gene Company.

Bioinformatic analyses were performed using various online tools and software: ExPASy (https://www.expasy.org/) was used to predict amino acid composition, molecular formula, molecular weight, isoelectric point, stability, and hy-

drophilicity; PSORT (http://psort1.hgc.jp/form.html) for subcellular localization prediction; SignalP (http://www.cbs.dtu.dk/services/SignalP/) for signal peptide analysis; TMHMM (http://www.cbs.dtu.dk/services/TMHMM/) for transmembrane domain prediction; DNAMAN for amino acid sequence alignment; and MEGA7 for phylogenetic analysis.

Gene Expression Analysis

Quantitative PCR (qPCR) was performed using the SYBR® Select Master Mix kit on the Mx3005P Real-Time PCR System. The *Actin* gene served as the internal reference with primers ACT-F (5 -CTGGTGTTATGGTTGGGATGG-3) and ACT-R (5 -GGGGTGCCTCGGTAAGAAG-3). The target gene primers were GE3-F (5 -CATCTGGACTGCGAAACTATCC-3) and GE3-R (5 -GCTGGTGATGGTGACGTTAAT-3). The 20 L qPCR reaction contained 10 L 2×SYBR mix, 1 L each of forward and reverse primers (10 mol · L ^{1}), 1 L cDNA template, and 7 L ddH O. The qPCR cycling conditions were: 95°C for 10 min; 40 cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s.

Results

Cloning of IbGL3

Based on the *IbGL3* gene EST sequence obtained from sweet potato transcriptome data, specific primers were designed to amplify the full-length sequence, yielding a specific band of approximately 2,100 bp [Figure 1: see original paper]. The target fragment was ligated into the T-vector for sequencing, and the results were consistent with the transcriptome sequencing data. The cloned *IbGL3* gene had a total length of 2,120 bp, containing a complete open reading frame of 1,878 bp.

Molecular Characteristics of IbGL3

The IbGL3-encoded protein consists of 625 amino acids, with serine (Ser) being the most abundant at 63 residues (10.1%). The molecular formula of IbGL3 protein is C H N O S , with a predicted molecular weight of 69.08 kDa and a theoretical pI of 5.20. The instability index of 43.06 classifies it as an unstable protein, while the grand average of hydropathicity of -0.555 indicates it is hydrophilic. Subcellular localization prediction revealed that IbGL3 is most likely localized in the nucleus, with a nuclear localization signal (PSSKKRKASKT) at the C-terminus. Signal peptide analysis showed that IbGL3 lacks a signal peptide and is not a secretory protein, and transmembrane domain analysis confirmed it has no transmembrane structures.

Evolutionary and Homology Analysis of IbGL3

To further understand the function and evolutionary characteristics of IbGL3, its amino acid sequence was clustered with different *Arabidopsis* bHLH subgroups and other plant bHLH proteins identified as involved in anthocyanin synthesis. The results showed that IbGL3 clusters with TT8, GL3, and EGL3, belonging to the IIIf subgroup [Figure 2: see original paper]. Most bHLH proteins regulating anthocyanin biosynthesis identified in various plants belong to the IIIf subgroup, suggesting that IbGL3 may function in regulating anthocyanin biosynthesis in sweet potato. Sequence alignment of IbGL3 with other anthocyanin-regulating bHLH proteins revealed that these proteins all contain three conserved domains: the MIR region at the N-terminus that interacts with MYB proteins, the bHLH domain, and an ACT-like domain at the C-terminus [Figure 3: see original paper].

Correlation Analysis Between IbGL3 Expression and Anthocyanin Accumulation

To analyze the correlation between *IbGL3* expression and anthocyanin accumulation, quantitative PCR was used to detect the expression patterns of *IbGL3* in storage roots of different sweet potato varieties. The results showed that *IbGL3* expression was highest in purple-fleshed sweet potato, moderate in light purple-fleshed varieties, and lowest in white-fleshed sweet potato, demonstrating a positive correlation with anthocyanin content [Figure 4: see original paper].

Discussion and Conclusion

The bHLH transcription factor family represents one of the most widespread classes of transcription factors in eukaryotes, playing crucial regulatory roles in various physiological processes including plant cell size and fate determination, hormone responses, metal homeostasis, photomorphogenesis, and floral organ development (Heim et al, 2003; Feller et al, 2011; Yang et al, 2017). Most bHLH transcription factors regulating anthocyanin biosynthesis belong to the IIIf subgroup, and modification or overexpression of anthocyanin-related bHLH genes can affect anthocyanin accumulation in plants (Xu et al, 2015; Li et al, 2016; Lim et al, 2017). This study cloned *IbGL3*, a bHLH gene homologous to *Arabidopsis* GL3, from sweet potato. The coding region spans 1,878 bp and encodes 625 amino acids. Subcellular localization prediction indicates nuclear localization, suggesting that IbGL3 functions in the nucleus. Phylogenetic analysis revealed that IbGL3 belongs to the IIIf subgroup of bHLH transcription factors and shares high homology with anthocyanin-related bHLH proteins from other plants, containing conserved MIR, bHLH, and ACT domains. bHLH transcription factors typically interact with MYB transcription factors to coordinately regulate anthocyanin biosynthesis, with the MIR region at the N-terminus serving as the MYB interaction domain (Heim et al, 2003; Feller et al, 2011). The

bHLH domain is a DNA-binding domain that can form homodimers or heterodimers, which is prerequisite for DNA binding (Heim et al, 2003; Feller et al, 2011). The ACT-like domain at the C-terminus is rich in acidic amino acids and can bind RNA polymerase II to initiate transcription; this region is also involved in bHLH dimer formation (Feller et al, 2006). Functional prediction analysis suggests that IbGL3 may regulate anthocyanin biosynthesis in sweet potato. Expression analysis demonstrated that *IbGL3* is highly expressed in purple-fleshed sweet potato, moderately expressed in light purple-fleshed varieties, and weakly expressed in white-fleshed sweet potato, showing positive correlation with anthocyanin accumulation. These findings further support that IbGL3 is likely a regulatory gene for anthocyanin biosynthesis in sweet potato. Future studies will investigate the biological function and regulatory mechanism of IbGL3 in anthocyanin biosynthesis, providing a foundation for genetic improvement of anthocyanin content in sweet potato.

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