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Full Length Research Paper

The anthocyanidin synthase gene from sweetpotato [*Ipomoea batatas* (L.) Lam]: Cloning, characterization and tissue expression analysis

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Anthocyanidin synthase (ANS) catalyzes the biosynthesis of anthocyanidin, which is a late gene for anthocyanin biosynthesis. In order to investigate the role of anthocyanidin synthase in anthocyanin biosynthesis, we cloned and characterized the anthocyanidin synthase gene from purple-flesh sweet potato (*Ipomoea batatas* (L.) Lam) Yuzi 263, which was designated as *IbANS*. The cDNA fragment of the ANS gene of sweet potato was 1375-bp in length which contained a 1086-bp open reading frame that encoded a 362-amino acid polypeptide. Comparative analysis showed that *IbANS* had a high similarity to other plant ANSs. The tissue expression profiles of *IbANS* indicated that it could be expressed in all tissues but at different levels. The higher expression level of *IbANS* was found in diameter (3.0 cm) of tuberous roots and periderms, while the lower expression level of *IbANS* was found in other tissues just coinciding with the anthocyanin content distribution.

Key words: Sweet potato, purple-fleshed, anthocyanidin synthase, cloning, characterization, expression profile.

INTRODUCTION

Anthocyanins are the important plant pigments visible to the human eyes for the coloring of plant organs, which belong to the widespread class of phenolic compounds collectively named flavonoids. They can act as anti-oxidants, phytoalexins or as antibacterial agents (Jin et al., 2003). Anthocyanins may be important factor along with other flavonoids in the resistance of plants to insect attacks (Harborne, 1988). Anthocyanins also possess known pharmacological properties and are used by humans for therapeutic purposes. Kamei et al. (1998) reported that anthocyanins had anti-tumor activity, and they found that the anthocyanin fraction from red wine suppressed the growth of HCT-15 cells and AGS cells, which were derived from human colon cancer and human gastric cancer respectively.

The anthocyanin biosynthesis pathway is well-known for secondary products (Dixon and Steele, 1999). It has been extensively studied in the flowers of petunia *(Petunia hybrida)* and snapdragon *(Antirrhinum majus)*, and in the kernels of maize *(Zea mays)* (Holton and Cornish, 1995). Among the genes and enzymes examined in this pathway, anthocyanidin synthase (ANS) is an important component because it converts leucoanthocyanidins to anthocyanidins, the first colored compound in the anthocyanin pathway (Springob et al., 2003). In *Forsythia,* ANS transcripts have been detected in the sepals, but not in the anthers or petals (Rosati et al., 1999). Expression of the *Perilla* ANS gene, which is detected in red leaves of the red forma, is coordinately induced in the forma by high-intensity white light (Gong et

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al., 1997). Likewise, transcripts of the apple ANS gene are detected preferentially in the skin tissues of red fruits, but not green and are also coordinately induced by light (Kim et al., 2003).

Sweet potato [*Ipomoea batatas* (L.) Lam] is grown in more than 100 countries as a valuable source of food, animal feed and industrial raw material. Sweet potato plays an important role in household food security in the eastern African countries where small-scale farmers mainly women grow sweet potato (Luo et al., 2006). The major coloring constituents in sweet potato, specifically in purple-fleshed varieties, have been identified as acylated anthocyanins (Imbert et al., 1966; Zulin et al., 1992; Terahara et al., 1999). Although ANS from sweet potato was reported in some cultivars (Zhou et al., 2010; Lalusin et al., 2006), they have not been clarified as the anthocyanin biosynthesis pathway and transportation process and ANS sequence may also be different in different cultivars. In the present study, we used the methods of rapid amplification of cDNA ends (RACE) to isolate the cDNA encoding ANS from purple-fleshed sweet potato (*I. batatas* (L.) Lam). Some expression analyses had been conducted in different tissues of sweet potato including roots, leaves and stems by real-time quantitative PCR, and detected the tissue distribution of purple pigments at the same time.

MATERIALS AND METHODS

Plant materials, enzymes and strains

All tissue materials including roots, stems and leaves were excised from purple-fleshed sweet potato Yuzi 263 grown in the sweet potato Research Center of Southwest University (Chongqing, China). Plant tissues were immersed in liquid nitrogen immediately after excision and preserved in a -70℃ ultra low temperature refrigerator for RNA extraction. And at the same time, the same tissues were used to measure the anthocyanin content. *Escherichia coli* DH5α strain (Invitrogen, Netherlands) was used as host cells for the molecular operation. The plasmid pMD19-T vector (TaRaKa, Japan) was used for subcloning for sequence.

RNA isolation

The RNA isolating kit provided by TianGen (Beijing, China) was used for total RNA extraction of the sweet potato tissues. The quality and concentration of the RNA was checked by agarose gel electrophoresis (EC250-90, E-C Apparatus Corporation) and spectrophotometer (WFZUV-2100, UnicoTM (Shanghai, China) Instruments Inc.) analyses and the RNA samples were stored in a -70℃ ultra low temperature refrigerator prior to RACE and real-time quantitative PCR.

Cloning of the full length cDNA of ANS

The total RNAs of sweet potato root were reversely transcripted in a volume of 50 µl according to the instruction manual of TaKaRa RNA PCR Kit (AMV) Ver.3.0. Then, a core fragment of *IbANS* was isolated with a pair of primers f*ibans*: 5'-GTGGACTTGAAAGGGATCAAC-3'; r*ibans*: 5'-GCCTGAAGAGCTTATGCTTAAT-3') from the total cDNAs of sweet potato roots. The PCR was carried out by denaturation of the DNA at 94℃ for 5 min (min), which was followed by 30 cycles of amplification (94℃ for 45 s, 55℃ for 45 s and 72℃ for 1 min) and by extension at 72℃ for 8 min. The amplified DNA fragment of the core fragment of *IbANS* was subsequently purified and cloned into pMD19-T Vector (TaKaRa, Japan). The sequence was completed by Genscript Company (China). The gene-specific primers (GSP3-1) were deduced from the core sequence of *IbANS*. The 3' RACE was carried out essentially according to the manufacturer's instructions using the kit from Takara. The cDNA was used as a template in first round PCR in the presence of the primer M13, primer M4 (TaKaRa) and GSP3-1 (5'- GGCAAGTACAAGAGCATTCT-3'). For the second round PCR, 5 µl of the diluted first round PCR products were re-amplified with M13 Primer M4 (TaKaRa) and GSP3-2 (5' -AAGGAAAAGATCATTCTCCAGC-3'). For 5' RACE, the *IbANS* gene-specific primers (GSP5-1) were designed according to the core sequence. Firstly, 5' RACE-ready cDNA were obtained by BD SMART™ RACE cDNA Amplification Kit (USA) and as the template for the first round PCR, the UPM (provided by the kit) and GSP5-1 (5'-CATACTTCTCTTTCTCCTCAATTG-3') were used as the primers to be amplified using BD Advantage™ 2 PCR Kit (USA). For the second round PCR, 2 µl of the diluted first round PCR product was re-amplified with NUP (SMART™) and GSP5-2 (5'-GTATCCCGTGGTTGACCAGGTGCAT-3'). A deduced full-length cDNA of *IbANS* was obtained by assembling the 3' end, the core fragment and 5' end. Then, the coding sequence of *IbANS* was amplified through proof-reading PCR using a pair of primers, ANS-F (5'- ATGGTGACTACTATTACTGTTCCG-3') and ANS-R (5' -TCATTCATCAGGTTTGGGCGTG-3'). The PCR products were subcloned into the pMD19-T vector (TaKaRa, Japan) and were introduced into *E. coli* DH5α cells. The clones were used for sequencing analysis. When confirmed by sequencing, the cDNA sequence of *IbANS* was submitted to GenBank.

Tissue expression profile of ANS by real-time quantitative PCR

Total RNAs isolated from different tissues including fibrous roots (FR), diameter of 0.5 cm tuberous roots (0.5 TR), diameter of 3.0 cm tuberous roots (3.0 TR), periderms (PD) , knots of stems (KS), stems(S), petioles (PT), leaves (L), shoot apexes (SP) were subjected to produce cDNAs by oligo(dT)18 primer using the first-strand cDNA synthesis kit (TakaRa, Japan) according to the protocol suggested by the manufacturer. Gene expression analysis was measured by real-time quantitative PCR, using the SYBR green I method on a Mastercycler ep realplex real-time cycler (Eppendorf, Germany). The real-time quantitative PCR was carried out with the SYBR® *Premix Ex Taq*™ II (Perfect Real Time) kit (TaKaRa, Japan). The real-time quantitative PCR thermal cycling conditions were 95℃ for 30 s, followed by 95℃ for 5 s 60℃ for 30s for 40 cycles, followed by a melt cycle 95℃ for 15 s, 60℃ for 30 s, and 95℃ for 15 s. The primers Qf*ibans*2 (5'-GGAAGGAAGGCTGGAGAAG-3') and Qr*ibans*2 (5'-TTGTGGAGGATGAA GGTGAG-3') were used for real-time PCR to amplify a 150-bp fragment of *IbANS* from different types of cDNAs. The 18S rRNA gene was chosen for normalization of gene expression. The products of real-time quantitative PCR were confirmed by the determination of the melt curves for the products at the end of each run by analysis of the products using agarose gel electrophoresis. Quantification of the gene expression was done with the comparative threshold (Ct) method (Heid et al., 1996). The experiments were repeated three times on independently isolated mRNA preparation.

Measurement of anthocyanin quantification

Extraction and measurement of anthocyanins were performed following the protocols of Zhang et al. (2006). The fresh plant samples

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   1 acgcggggatattttccatttatatatatataatcctctacacaatcaagatcgaatgaaaatcaagaaataatataatataattcaaca
  91 ATGGTGACTACTATTACTGTTCCGAGCAGGGTGGAGAGGCTAGCCGGCAGCGGCATCGAACGCATCCCGAAGGAGTACATAAGGCCGGAG
      M  V  T  T  I  T  V  P  S  R  V  E  R  L  A  G  S  G  I  E  R  I  P  K  E  Y  I  R  P  E
 181 GAAGAGCGGCCGAGCATCGGCGACATTTTTGCGGAGGAAAAGATGGGCGGAGGGCCACAAGTCCCGACCGTGGACTTGAAAGGGATCAAC
      E  E  R  P  S  I  G  D  I  F  A  E  E  K  M  G  G  G  P  Q  V  P  T  V  D  L  K  G  I  N
 271 TCGGAGGACTTGGAGGTGCGGGAGAAGTGCCGGGAGGAGCTGCGGAAGGCGGCGGTGGACTGGGGCGTCATGCACCTGGTCAACCACGGG
      S  E  D  L  E  V  R  E  K  C  R  E  E  L  R  K  A  A  V  D  W  G  V  M  H  L  V  N  H  G
 361 ATACCGGAGGAGCTCACCGGCCGCGTCAAGGCCGCCGGAGAGGGGTTTTTCGGTCAGCCAATTGAGGAGAAAGAGAAGTATGCTAATGAC
      I  P  E  E  L  T  G  R  V  K  A  A  G  E  G  F  F  G  Q  P  I  E  E  K  E  K  Y  A  N  D
 451 CAGGCGGCGGGGAATGTGCAGGGCTATGGGAGTAAGCTGGCAAATAATGCTAGTGGGCAGCTTGAGTGGGAGGATTACTTTTTCCACTGT
      Q  A  A  G  N  V  Q  G  Y  G  S  K  L  A  N  N  A  S  G  Q  L  E  W  E  D  Y  F  F  H  C
 541 GTTTTCCCTGAAGATAAGACTGATCTCTCCATTTGGCCTAAAACTCCTTCTGATTACATAGCTGCAACAAGGGAATATGCAAAGCAACTG
      V  F  P  E  D  K  T  D  L  S  I  W  P  K  T  P  S  D  Y  I  A  A  T  R  E  Y  A  K  Q  L
 631 AGGGCATTAACCACAACGGTGTTAGCAGTGCTGTCACTAGGGCTAGGGTTAGAGGAAGGAAGGCTGGAGAAGGAAGTGGGAGGGATGGAA
      R  A  L  T  T  T  V  L  A  V  L  S  L  G  L  G  L  E  E  G  R  L  E  K  E  V  G  G  M  E
 721 GAGCTTCTTCTGCAAATGAAAATAAACTACTACCCGAAATGCCCTCAGCCGGAGCTAGCTCTCGGCGTGGAAGCTCACACCGACGTCAGC
      E  L  L  L  Q  M  K  I  N  Y  Y  P  K  C  P  Q  P  E  L  A  L  G  V  E  A  H  T  D  V  S
 811 GCCCTCACCTTCATCCTCCACAACATGGTCCCGGGCCTGCAGCTCTTCTACGAGGGAAAATGGGTGACCGCCAAGTGCGTCCCTAACTCC
      A  L  T  F  I  L  H  N  M  V  P  G  L  Q  L  F  Y  E  G  K  W  V  T  A  K  C  V  P  N  S
 901 ATCATCATGCACGTGGGCGACACCGTCGAGATCCTCAGCAACGGCAAGTACAAGAGCATTCTCCACAGAGGGGTTGTGAACAGGGAGAAG
      I  I  M  H  V  G  D  T  V  E  I  L  S  N  G  K  Y  K  S  I  L  H  R  G  V  V  N  R  E  K
 991 GTGAGGGTTTCTTGGGCGGTGTTCTGTGAGCCGCCCAAGGAAAAGATCATTCTCCAGCCCCTGCCGGAGACCGTGTCTGAGGCGGACCCG
      V  R  V  S  W  A  V  F  C  E  P  P  K  E  K  I  I  L  Q  P  L  P  E  T  V  S  E  A  D  P
1081 CCGCGCTTCCCGCCCCGCACTTTTGCCCAGCACATTAAGCATAAGCTCTTCAGGCAAACTGATCAGGAAGCAGCTGACACGCCCAAACCT
      P  R  F  P  P  R  T  F  A  Q  H  I  K  H  K  L  F  R  Q  T  D  Q  E  A  A  D  T  P  K  P
1171 GATGAATGAtgatgatcagcattcaaattagcttattcctttgggaaaatatatagtaaaacattggttaatatgatgtttcaaatctac
      D  E  *
1261 tattagaatgtaatttgagctttgtggagtccatttggtattataatgggttgtttttataacaatcgctttattaaagcagctcaacac
1351 tgccgttaacaaaaaaaaaaaaaaa
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Figure 1. The full-length cDNA and deduced amino acid sequences of purple-fleshed sweet potato (*I. batatas*) anthocyanidin synthase (*IbANS*). The deduced amino acid and the corresponding cDNA sequences were indicated in capital letters. The start codon (ATG), the stop codon (TGA) and the poly (A) tail were underlined, the stop codon (TGA) was marked with an asterisk; the 5`- and 3`-untranslated regions were shown in lower case letters.

(2 g) were ground using 15 ml citric acid-disodium hydrogen phosphate buffer (pH 3.0) and then added to the final volume of 50 ml. Samples were incubated for 3 h at 25℃ in an orbiter (100 rpm). After centrifugation (8000 g at 25℃ for 5 min), the supernatant that contained the purple anthocyanins was used for optical asorbrance measurement at 530 nm (U-1100, HITACHI Co., Japan) to determine the anthocyanin content. Each data represents the average of three independent experiments. Error bars indicate the standard errors of the average of the anthocyanin content.

RESULTS AND DISCUSSION

Cloning of the full-length cDNA of *IbANS*

Based on the alignment of the ANS sequences from other plants, the primers f*ibans* and r*ibans* were designed to amplify a core fragment of the ANS gene from purple-fleshed sweet potato. The PCR product was 886 bp in length. Then, gene-specific primers based on the amplified core fragment of *IbANS* were designed for the amplification of the 5' and 3' ends of cDNA by 5'-RACE and 3'-RACE. Two fragments of 364 bp 5' end and 349 bp 3' end of cDNA sequences were obtained by 5'-RACE and 3'-RACE, respectively. By assemblage of the sequences of 3' end, 5' end and the core fragment on ContigExpress, the full-length cDNA sequence of *IbANS* was generated which was 1375 bp. Then, the coding sequence of *IbANS* was amplified through proof-reading PCR to confirm the full-length cDNA sequence. Finally, an integrity cDNA sequence of *IbANS* was obtained, which was 1,375 bp in length with 90-bp 5' untranslated region (UTR), 1086-bp coding sequence and 199-bp 3' UTR including 15-bp poly-A tail (Figure 1). The deduced amino acid (aa) sequences indicate that *IbANS* encodes a protein of 362 aa long. The full-length cDNA was tested for similarity to other cloned ANS genes using BLAST. The sequence comparative analysis showed that it was highly similar with the other ANSs from plant species, such as *Ipomoea nil* (94% identities, accession number: AB073920), *Ipomoea purpurea* (95% identities, EU032614), *Ipomoea horsfalliae* (95% identities, GQ180934) and *Solenostemon*

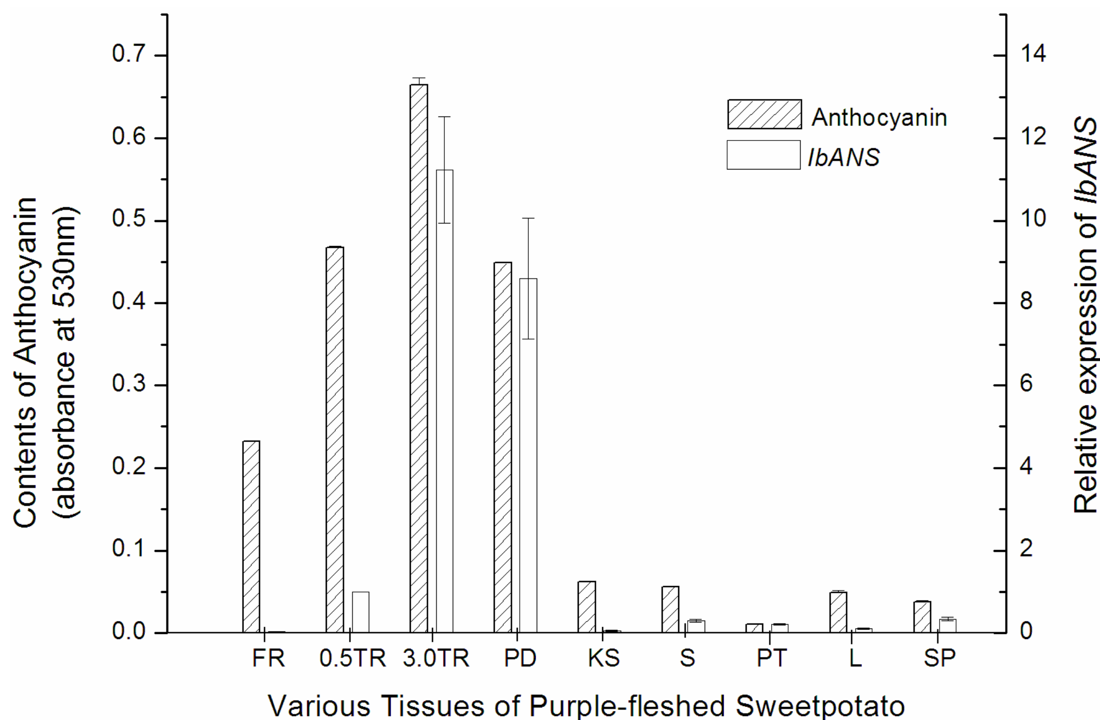

Figure 2. Anthocyanin accumulation and gene expression of *IbANS* in different tissues of purple-fleshed sweet potato Yuzi 263. Contents of anthocyanin (Left) was determined by optical asorbrance measurement at 530 nm and relative expression (Right) of *IbANS* was measured by real-time quantitative PCR in the fibrous roots (FR), diameter of 0.5 cm tuberous roots (0.5 TR), diameter of 3.0 cm tuberous roots (3.0 TR), periderms (PD), knots of stems (KS), stems (S), petioles (PT), leaves (L) and shootapexes (SP). Each data was presented as the means ± SD from three replicates.

scutellarioides (80% similarity, EF522159). These high identities suggested that *IbANS* was structurally a member of the ANS family and a new gene from purple-fleshed sweet potato Yuzi 263. Then, *IbANS* was submitted to GenBank and assigned an accession number: GU598212.

Anthocyanin accumulation and tissue expression profile

We determined total anthocyanin content of the fibrous roots (FR), diameter of 0.5 cm tuberous roots (0.5 TR), diameter of 3.0 cm tuberous roots (3.0TR), periderms, knots of stems, stems, petioles, leaves and shootapexes of 40-day-old purple-fleshed sweet potato plants (Yuzi 263). The anthocyanin content was relatively stable from 40 to120 days after planting in sweet potato Yuzi 263 (Fu et al., 2007). Using the same tissues, real-time quantitative PCR was used to examine the expression profile of *IbANS*. Results showed that the total anthocyanin content of diameter of 3.0 cm tuberous roots (3.0 TR) was the highest of the nine tested tissues, followed by diameter of 0.5 cm tuberous roots (0.5 TR) and periderms. The highest content in tissue (3.0 TR) was about 62 times compared to the lowest content in tissue petioles (Figure 2). Results also indicated that *IbANS* was expressed in all examined tissues but was highest in diameter of 3.0 cm tuberous roots (3.0 TR) like with the situation of anthocyanin contents in the tissues (Figure 2). The expression level of *IbANS* was much higher in periderms than that in the fibrous roots, knots of stems, stems, petioles, leaves and shootapexes showing very low correlation with the anthocyanin content of these tissues which strongly suggested that high expression of *IbANS* caused the high content of anthocyanin in diameter of 3.0 cm tuberous roots (3.0 TR) or periderms and then the anthocyanin was transferred to others tissues. In most plant species, anthocyanin production is limited to certain tissues and occurs during specific developmental stages. The visible accumulation of these compounds usually reflects the activity of the biosynthetic enzymes functioning in the pathway (Koes et al., 1994). In most plants, pigmentation is limited to some specific tissues and this is regulated by external and internal factors (Koes et al., 2005). Most of the regulation of pigment synthesis occurs via the coordinated transcriptional control of the structural genes (Mol et al., 1998). The results obtained in this study provide additional evidence that the expression of the anthocyanin pathway gene *IbANS* was specifically upregulated in certain plant parts (diameter of 3.0 cm tuberous roots). In

summary, cloning and characterization of the *IbANS* gene from purple-fleshed sweet potato facilitates the understanding of the molecular mechanisms of anthocyanin biosynthesis and provides an important gene of interest to metabolic engineering in the anthocyanin biosynthetic pathway in sweet potato (*I. batatas* (L.) Lam).

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