DOI: 10.1007/s10535-015-0502-5 BIOLOGIA PLANTARUM 59 (2): 325-330, 2015

Anthocyanin accumulation rate and the biosynthesis related gene expression in *Dioscorea alata*

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Abstract

In this study, the anthocyanin content and real-time quantitative expression of the anthocyanin biosynthesis-related genes were investigated in leaves, stems, and tubers of purple yam (*Dioscorea alata* L.). The anthocyanin content, its accumulation, and the expression of genes encoding phenylalanine ammonia lyase (PAL), flavanone-3-hydroxylase (F3H), anthocyanidin synthase (ANS), and UDP-glycose flavonoid glycosyl transferase (UFGT) were studied. The anthocyanin content in the leaves and stems was high at early stages of growth, but it decreased and remained at a similar level from the 35th day onward. The anthocyanin content in the tubers firstly increased, reached a high peak at the 110th day of growth, after which decreased. Anthocyanin accumulation rates and the expressions of the anthocyanin biosynthesis genes were high at the early stages of growth in the leaves and stems, but in tubers, two peaks were observed: at days 80 and 140 for the gene expression and at days 125 and 170 for the anthocyanin accumulation rate. Thus, there was coordination between the gene expressions and the anthocyanin accumulation rates in the various organs as well as in the entire plants.

Additional key words: anthocyanidin synthase, flavanone 3-hydroxylase, phenylalanine ammonia lyase, plant ontogeny, purple yam, UDP-glycose flavonoid glycosyl transferase.

Introduction

The purple yam (*Dioscorea alata* L.) has the following known benefits: toning the kidneys, nourishing the stomach, refreshing and enriching the saliva, and aiding the lungs. Therefore, it can be used as both a medicinal and edible plant and it is widely cultured in South Pacific and southern China (Champagne *et al*. 2011). Purple yam anthocyanin extract has a strong antioxidant capacity and a free radical scavenging capacity (Zhang *et al*. 2013) which has caused to attract attention from various industries in recent years (Graf *et al*. 2013, Meng *et al.* 2014).

The biosynthesis pathway of the anthocyanin is controlled by genes, such as phenylalanine ammonia lyase (PAL), chalcone synthase (CHS), flavanone-3-hydroxylase (F3H), anthocyanidin synthase (ANS), dihydroflavono-4-reductase (DFR), and UDP-glycose flavonoid glycosyl transferase (UFGT), and is regulated by some positive and negative transcription factors (Aguilar-Barragán and Ochoa-Alejo 2014). Kim *et al*. (2003) found that the expressions of *F3H*, *DFR*, *ANS*, and *UFGT* genes in apple peels are up-regulated, and the anthocyanin starts to accumulate after removing the bags which are employed for preventing pathogen infection. Hara *et al*. (2004) demonstrated the *PAL*, *F3H*, *DFR*, and *ANS* genes expression in red radish seedlings, and also the anthocyanin content are up-regulated after addition of sucrose in the medium. Szankowski *et al*. (2009) silenced *ANS* by RNAi technology and found that the synthesis of anthocyanin in apple peels is blocked, which imply that the *ANS* gene plays an important role in the anthocyanin biosynthesis. In recent years, the anthocyanin biosynthesis in underground organs has become a hot topic of research (Liu *et al*. 2010). However, in comparison with the research on *Arabidopsis*, petunia, and other model plants (Debeaujon *et al*. 2003,

Submitted 28 May 2014, *last revision* 11 October 2014, *accepted* 30 October 2014.

Abbreviations: ANS - anthocyanidin synthase; CHS - chalcone synthase; F3H - flavanone-3-hydroxylase; DFR - dihydroflavonol-4-reductase; PAL - phenylalanine ammonia lyase; qRT-PCR - quantitative real-time PCR; UFGT - UDP-glycose flavonoid glycosyl transferase.

Acknowledgements: This work was supported by the Jiangsu Agricultural Science and Technology Innovation Fund [No.CX(13)5008], China.

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Lepiniec *et al*. 2006, Feyissa *et al*. 2009), little research on the purple yam has been conducted.

In this study, the anthocyanin content, the accumulation rate, and the spatial and temporal expressions of biosynthesis-related genes in leaves, stems, and tubers of purple yam were investigated.

Materials and methods

Plants: Rhizomes of purple yam (*Dioscorea alata* L.) cv. Yzi006 (one of the cultivars rich in anthocyanin) were planted in an experimental field at the Jiangsu Academy of Agricultural Sciences on April 2, 2013. Sprouting began on April 26, 2013. Fresh leaves, stems, and tubers collected after the 5th, 20th, 35th, 50th, 65th, 80th, 95th, 110th, 125th, 140th, 155th, and 180th day were immediately frozen in liquid nitrogen. The samples were then stored at -80 °C until being utilized for the determination of anthocyanin and mRNA content. Two plants were regarded as a repeat, and three repeats were collected for every sample.

Measurement of the anthocyanin: The anthocyanin content was measured by the modified method of Mehrtens *et al*. (2005). Fresh tissues were cut into pieces and incubated with 6 cm^3 of an extraction solution [concentrated HCl + 80 % (v/v) ethanol 1: 99] for 1 h, and then the absorbances at 530 and 657 nm were measured on a UV-VIS spectrophotometer (*ND752*, *SPSIC*, Shanghai, China). The anthocyanin content (Q) was calculated as: $Q = (A_{530} - 0.25 \times A_{657})/FM$, where FM is the fresh mass of the sample. All samples were measured in triplicates. For statistical analyses and graphics, *Microsoft Office Excel* version *2010* was used.

The relative amount of accumulated anthocyanin (A) was calculated as described by Hou *et al.* (2010) according to an equation $A_n = Q_n \times FM_n$, where n is the series number indicating the sampling time in Table 2, Q_n and FM_n are the anthocyanin content and the fresh mass, respectively. The relative rate of anthocyanin accumulation (R) was calculated by using an equation $R = (A_n - A_{n-1})/D$, where D is the interval between the two samplings (15 d) (Hou *et al.* (2010).

Isolation of related genes: RNA was isolated and first strand cDNA was synthesized using the modified method of Valderrama-Cháirez *et al*. (2002). By utilizing coding sequences which are known in crops of the *Actin-2*, *PAL*, *F3H*, *ANS*, and *UFGT* genes in accordance with the NCBI database, the conserved regions of the corresponding gene sequences were obtained. PCR primers were designed by comparing them with the EST database of the yam genome. Target genes *Actin-2*, a forward primer (F): 5'-ATTTGGCACCATACTTTC-3', a reverse primer (R): 5'-TTGCCATACAGATCCTTC-3'; *PAL* F: 5'-TAACTCCTTGGGCTTGATTTCTTC-3', R: 5'-CACCTTCCCCTGGCTGATG-3'; *F3H* F: 5'-GCC TGGCCTGCACACTACTTG-3', R: 5'-CTACCCATT TTCCGGCGATACA-3'; *ANS* F: 5'-TGGCCTGCA GGTCTTCTACGA-3', R: 5'-AACGGACGCAGCAG CACCTT-3'; and *UFGT* F: 5'-GTGTGCCGTTGA TATGTA-3', R: 5'-GTGCTGTTGTCTTGAGTT-3'.

The PCR amplification reaction solution (50 mm^3) consisted of 3 mm^3 of a cDNA template, 5 mm^3 of a 10× buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$), 5 mm^3 of 2.5 μM dNTPs, 4 mm^3 of a forward primer (10 μM), 4 mm^3 of a reverse primer (10 μM), and 29 mm^3 of ultra-pure water (ddH_2O). The amplification procedure was as follows: a pre-degeneration at 94 °C for 2 min, then 94 °C for 40 s, 56 °C for 40 s, and 72 °C for 60 s for 35 cycles; and an extension at 72 °C for 10 min. PCR products were retrieved by using agar gel electrophoresis. The DNA sequence and pMD-18 T vector were incubated at 16 °C for 12 h for ligation. After the transformation in the DH5α, strains were cultured by using a Luria-Bertani broth. The strains were then identified by using universal primers M13F (-47) (5'-CGCCAGGGTTTTCCCAGT CACGAC-3') and M13R (-48) (5'-GAGCGGATA ACAATTTCACACAGG-3').

Real-time RT-PCR analysis: Online sequence alignments were conducted on the obtained objective fragment sequences of the genes *Actin-2, PAL, F3H, ANS*, and *UFGT* using the *BLAST* software which displayed the high similarity with the related gene nucleotide sequences of other crops as well. Additionally, the related gene nucleotide sequence fragments of purple yam and the design of real-time quantitative PCR primers were determined. The primers for the real-time quantitative PCR were *Actin*-2 qF: 5'-GATTCTGGT GATGGAGTC-3', qR: 5'-TGGTGGTGAATGAGT AAC-3'; *PAL* qF: 5'-TGGCAACACTTCCATCTT-3', qR: 5'-CATTCTCATAGGCAACCC-3'; *F3H* qF: 5'-CTTGTTACAGGACCAGAT-3', qR: 5'-CGACAT TCTTGAACCTAC-3'; *ANS* qF: 5'-ACTCCATTGTCG TCCA-3'), qR: 5'-ATCCTCACCTTCTCCTTAT-3'. The primer sequences of the *UFGT* qF/R were identical to the *UFGT* F/R primers. The real-time quantitative expressions of the various genes in different tissues were detected and analyzed on an *ABI7500* real-time PCR analyzer (*Application Biological System*, Foster City, USA). *Actin-2* was used as reference gene. All amples were measured in triplicates. The relative expression of the target genes to the *Actin-2* standard were given by the equation: $R_{(target/Actin-2)} = 2^{-\Delta\Delta Ct}$. ΔΔCt values were calculated as described by Yuan *et al.* (2006). Data are presented as means ± SD. Significances of differences were indicated at $\alpha = 0.05$ and $\alpha = 0.01$.

Results

The anthocyanin content in the leaves and stems was highest in the early stages of growth and then decreased remaining at a consistently low level after day 35. Tubers started to form at the 50th day after planting. The content of anthocyanin in the tubers at the early stages was high and then gradually decreased. However, after the 95th day of growth, the content of anthocyanin in the tubers increased rapidly and reached a maximum on day 110, followed by a decrease. The content of anthocyanin in the tubers was higher than in the leaves and stems throughout the entire growth period. The anthocyanin content in the leaves was higher than in the stems at the early stages of growth (Fig. 1).

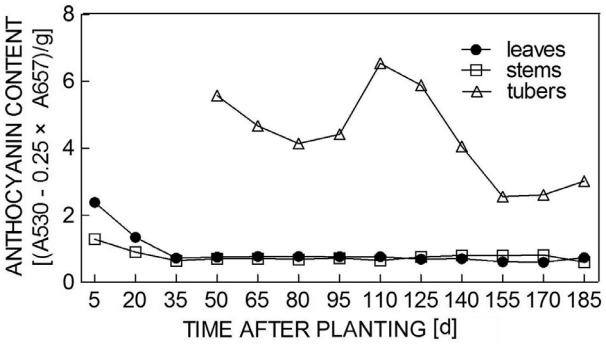

Fig. 1. The anthocyanin content in leaves, stems, and tubers of purple yam. The averaged data from triplicates.

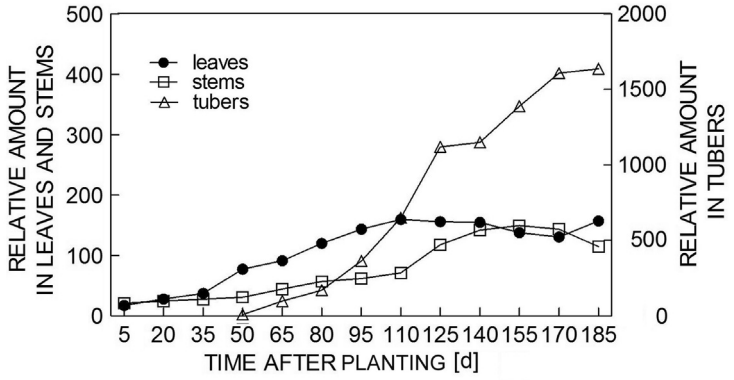

Fig. 2. A relative anthocyanin accumulation in leaves, stems, and tubers of purple yam. The averaged data from triplicates.

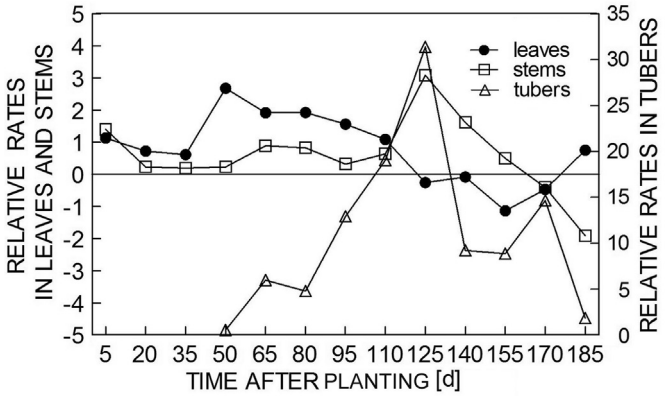

Fig. 3. Relative rates of anthocyanin accumulation in leaves, stems, and tubers of purple yam. The averaged data from triplicates.

On the 110th day of growth, the relative anthocyanin accumulation in the leaves reached its maximum after which it began to decline. The relative accumulation of anthocyanin in the stems reached its maximum on day 155. The relative accumulation of anthocyanin in the tubers increased throughout the whole growth period (Fig. 2).

The relative accumulation rates of anthocyanin in the leaves and stems were high at the early stages of growth and then began to decrease until the 35th day. After that, the relative accumulation rate of anthocyanin in the leaves began to increase and reached its maximum at day 50, but then it began to decrease at the point when the relative accumulation rate of anthocyanin in the stems and tubers began to increase. At day 125, when the accumulation rate in the stems and tubers reached a maximum, the accumulation rate in the leaves became negative and the content of anthocyanin began to reduce. These values in the stems became negative after day 170, whereas the relative accumulation rate in the tubers began to increase again (Fig. 3).

All of the obtained fragments were identified with the

Table 1. Expressions of anthocyanin synthesis genes in different tissues. Means ± SD, $n = 3$, *, ** - indicate significant differences at $P < 0.05$ and $P < 0.01$, respectively.

Organ	*PAL*	*F3H*	*ANS*	*UFGT*
Young leaves	20.07 ± 2.98**	28.42 ± 3.02**	103.46 ± 7.49**	96.91 ± 9.90**
Mature leaves	3.24 ± 0.37	0.03 ± 0.02	0.19 ± 0.02	0.84 ± 0.17
Young stems	99.91 ± 7.49**	7.39 ± 1.63**	1.21 ± 0.11**	1.23 ± 0.23*
Mature stems	1.99 ± 0.19	0.01 ± 0.00	0.01 ± 0.00	0.11 ± 0.03
Upper part of tubers	21.11 ± 2.27*	89.09 ± 7.97**	125.76 ± 16.00**	138.38 ± 2.91**
Lower part of tubers	14.41 ± 0.56	14.41 ± 3.36	10.93 ± 2.66	10.22 ± 0.45

corresponding sequences of the target genes in the yam genome, and they proved to be highly similar to the nucleotide sequences in other crops. Therefore, the real-time PCR primers of these genes were designed according to the obtained nucleotides.

The expressions of the anthocyanin biosynthesis genes were mainly observed in the young leaves and young stems (Table 1). The relative expressions of these genes in the mature leaves and mature stems were relatively low. The expressions of these genes were higher in the upper part of the tubers than in their lower part. Therefore, the expression analyses of the genes were focused on the young leaves, the young stems, and the upper parts of the tubers to avoid any redundant work.

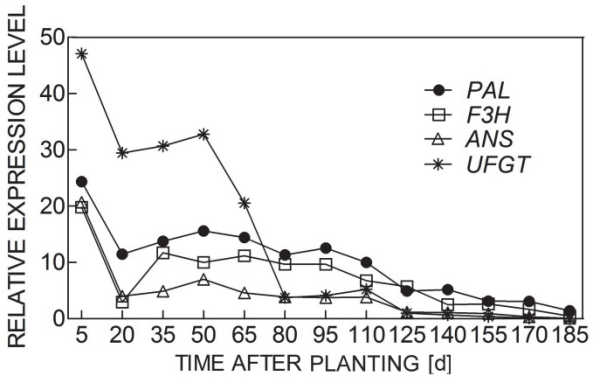

Fig. 4. Relative expressions of anthocyanin biosynthesis genes in the leaves of purple yam.

The expressions patterns of the *PAL, F3H, ANS*, and *UFGT* genes in the leaves were similar (Fig. 4). The expressions were highest on the 5th day after planting and decreased until the 20th day. The expressions increased again on day 50, and then decreased gradually. At the 125th day, when the accumulation rate of the anthocyanin in the leaves became negative (Fig. 3), the expressions of *PAL*, *F3H*, *ANS*, and *UFGT* were very low.

The expressions of the *PAL*, *F3H*, *ANS*, and *UFGT* genes in the stems were high in the early stages of growth (Fig. 5). Then the expressions of the *F3H*, *ANS* and *UFGT* decreased, whereas the maximum expression of the *PAL* gene appeared from the 50th to 110th day of

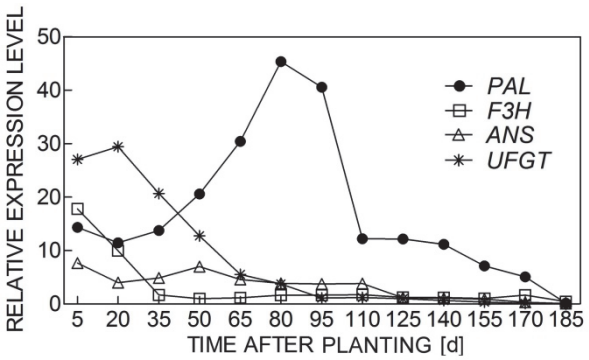

Fig. 5. Relative expressions of anthocyanin biosynthesis genes in the stems of purple yam.

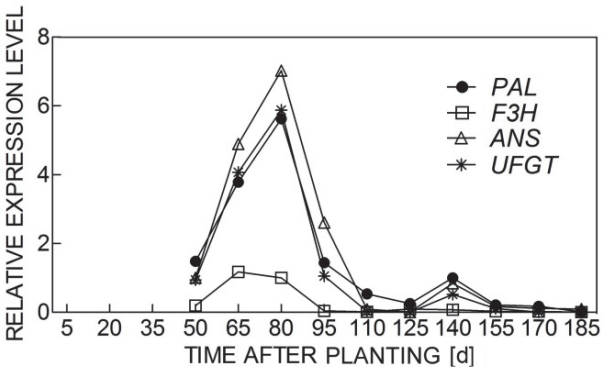

Fig. 6. Relative expressions of anthocyanin biosynthesis genes in the tubers of purple yam.

growth. After this stage, a rapid accumulation of the anthocyanin in the stems began, (Fig. 3) so the peak of the anthocyanin accumulation rate in the stems occurred later than the expression peak of the *PAL* gene.

The expressions of the *PAL*, *F3H*, *ANS*, and *UFGT* genes were high when the anthocyanin began to be synthesized in the tubers (Fig. 6). This period, between the 50th and 95th day of growth, was the first expression maximum, whereas the second maximum was on day 140, and then the gene expressions decreased. These two expression peaks corresponded with the two peaks of the anthocyanin accumulation rates (Fig. 3).

Discussion

The anthocyanin accumulation in the leaves, stems and tubers of the purple yam plants and dynamic changing the accumulation rates demonstrated that there was a large coordination between the anthocyanin amounts and the accumulation rates in the leaves, stems, and tubers. When the accumulation rate of anthocyanin in the leaves began to decline on the 50th day of growth, the accumulation rates of the anthocyanin in the stems and tubers began to increase rapidly. It was speculated that the above phenomenon is related to the coordination of plant metabolism as the plant itself was found to be able to manipulate the metabolism in different organs during development (Majdi *et al.* 2014).

The anthocyanin accumulation in the leaves of the purple yam plants agreed with dynamic trends of the relative expressions of the biosynthesis genes *PAL, F3H, ANS*, and *UFGT*. Also, the correlation between the changes of the accumulation rate of anthocyanin in the stems and the expression of the *PAL* gene was found, whereas no correlation with the *F3H, ANS*, and *UFGT* genes. The high expression of the *PAL* gene in the stems may be due to lignification of stems (Zhang *et al*. 2008, Gao *et al*. 2009, Kolahi *et al*. 2013), which suggests that stems are not the major organ for the anthocyanin synthesis and accumulation. During the period from the early stages of tuber formation (up to the 95th day of growth), there were high expressions of the *PAL*, *F3H*, *ANS*, and *UFGT* genes. The accumulation rate of anthocyanin in the tubers increased gradually to the first peak on the 125th day and to the second peak on the 170th day of growth, and it was closely related to the expressions of the anthocyanin synthesis genes.

The expression profiles of the *PAL*, *F3H*, *ANS*, and *UFGT* genes agreed with the expression levels of *PAL*, *F3H*, *ANS*, and *UFGT* genes described by Kim *et al*. (2003) and Hara *et al*. (2004), and also with results of Povero *et al*. (2011). Povero *et al*. (2011) transferred *Aft* and *Atv* genes into tomato plants, which up-regulated the expressions of the *PAL5*, *CHS*, *F3H*, *F3'H*, *DFR*, *ANS*, and *UFGT* genes, as well as the anthocyanin accumulation, and produced a purple fleshed tomato. In the present study, the expressions of the anthocyanin biosynthesis genes in the purple yam plants were correlated with the dynamic accumulation of anthocyanin.

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