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Full Length Research Paper

Cloning and expression trait of UDP-glucose:flavonoid 3-O-glucosyltransferase gene (UF3GT) in turnip

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Anthocyanin is a class of important secondary metabolites in plants. UDP-glucose:flavonoid 3-O-glucosyltransferase (UF3GT) is a committed catalytic enzyme in the late stage of anthocyanin biosynthesis. BrUF3GT1 and BrUF3GT2 genes were cloned by reverse transcription polymerase chain reaction (RT-PCR) method from 'Tsuda' and 'Yurugi Akamaru' turnips. The open reading frame (ORF) of BrUF3GT1 and BrUF3GT2 genes contained 1407 bp encoding proteins of 468 amino acids. Amino acid sequence analysis showed that BrUF3GT1 and BrUF3GT2 had 87% identity to UF3GT of *Arabidopsis thaliana*, and the glycosyltransferase protein family domain was in the amino acids sequence from 16 to 453. The nucleotide sequence of BrUF3GT1 and BrUF3GT2 genes showed only seven nucleotide differences, and one common deduced amino acid sequence. The northern blotting results showed that the expression of BrUF3GT1 and BrUF3GT2 genes could be induced by irradiation of ultra-violet A (UV-A), and the expression of the genes was correlated with light-exposure time. The 51.88 and 51.89 KDa proteins of BrUF3GT1 and BrUF3GT2 were successfully purified after prokaryotic induced expression.

Key words: Turnip, UDP-glucose:flavonoid 3-O-glucosyltransferase (UF3GT) gene, gene clone, sequence analysis, gene expression.

INTRODUCTION

Plant anthocyanins, a class of important flavonoids, accumulate in the vacuole of vascular plants and determine the pigmentation of flower, fruit and seed (Tanaka et al., 2008). The biosynthesis of anthocyanin is catalyzed by a group of enzymes. UDP-glucose:flavonoid 3-O-glucosyltransferase (UF3GT) is a critical catalytic enzyme in the late stage of anthocyanin biosynthesis. It produces anthocyanin glycosides by attaching glycosyl to unstable anthocyanins. Such glycosylated anthocyanins are stable and can be transported to the vacuole and stored (Kobayashi et al., 2001). At present, UF3GT genes from some plants have been cloned (Ford et al., 1998; Poudel et al., 2008; Li et al., 2010a; Kovinich et al., 2010). In plants, the biosynthesis of anthocyanins is not only closely related to the catalytic enzymes, but it also relates to the transcription factors (Ramsay and Glover, 2005; Niu et al., 2010; Ali et al., 2011; Paolocci et al., 2011).

Light not only provides the energy required for plant photosynthesis, but also provides the needed information for plants to adapt to surroundings and show normal growth. Anthocyanin biosynthesis of flower and fruit is closely related to light receptors and light signal transduction factors (Sheehan et al., 2004). Ultra-violet (UV) can regulate anthocyanin biosynthesis in plants (Guo et al., 2008). For instance, in soybean, the expression of genes that relate to catalytic enzymes of anthocyanin biosynthesis is up-regulated when irradiated by UV-B (Kim et al., 2008). Additionally, the expression of BrPAL1 and BrF3'H1 genes in 'Tsuda' turnip increased with the extension of UV-A treatment time (Xu et al., 2008a, b), which is synergetic with the increase of

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Abbreviations: UF3GT, UDP-glucose:flavonoid 3-O-glucosyltransferase; **RT-PCR,** reverse transcription polymerase chain reaction; **ORF,** open reading frame; **UV,** ultra-violet.

anthocyanin content. Currently, the mechanism of light-sensitive and light-insensitive anthocyanin biosynthesis is not yet clear.

Turnip (*Brassica campestris* L. ssp. Rapa) alias xiao man jing, is a subspecies of the *Brassica rapa* (Cruciferae). Root tubers of 'Tsuda' turnip need light to generate color and the irradiated part appears purple, while shielded parts appear white. However, root tubers of 'Yurugi Akamaru' turnip turn red regardless of light or darkness. That is to say, these root tubers can be colored in the dark. In this study, using 'Tsuda' and 'Yurugi Akamaru' turnips as tested materials, UF3GT genes from these two kinds of turnips were cloned using a reverse transcription polymerase chain reaction (RT-PCR) method and used to study expression under different irradiation regimes. Through northern blotting, the expression of UF3GT genes in 'Tsuda' and 'Yurugi Akamaru' turnips was detected under the condition of irradiation with UV-A. At the same time, UF3GT proteins from two kinds of turnips were purified by prokaryotic expression.

MATERIALS AND METHODS

Pure lines of 'Tsuda' and 'Yurugi Akamaru' turnips were cultivated in the greenhouse and the root tubers always grew in soil avoiding light irradiation. When the root tubers expanded after two months, we treated these root tubers for 0, 6, 18, 24 and 48 h, respectively, using UV-A of 352 nm (light intensity: 13.5 μmol•m-2s-1).

Primer sequence and reagents

In database, RT-PCR primer was designed on the UF3GT gene sequence of *Arabidopsis thaliana*. The primer sequence for RT-PCR was: F-5'- AAATGGGTGTTTTTGGATCG -3', R-5'- ATGAACTCATGACTTCACAAGTTC -3'. Primer sequence for probe synthesis was: F-5'- TGTTTGCTACAATACCGTCAGC -3', R-5'- GAAGAAAGAACCAATGCCCTC -3'. Primer sequence for constructing prokaryotic expression vector was: F1-5'- TGCAAATGGGTGTTTTTGGATCC-3', R1-5'- GCATGAACTCAT-GACTTCACAAGTTC -3', F2-5'- GGAATTCCATATGCAAA-TGGGTG -3' and R2-5'- TAAAAGTTGCGGCCGCATGAACTC -3'. As a result of introducing restriction sites and protective bases, the prokaryotic expression primers were too long, so two PCR reactions were needed to complete the full-length primer sequence.

LA-Taq DNA polymerase and DNA marker were purchased from TaKaRa Biotechnology (Dalian) Co., Ltd.; pBS-T vector was purchased from TIANGEN Biotech (Beijing) Co., Ltd.; PowerScriptTM reverse transcriptase was purchased from Clontech Co. Ltd.; the part reagents used for northern blotting were purchased from Roche Co. Ltd.; protein purification column for prokaryotic expression was obtained from GE Co. Ltd.

Cloning of full-length cDNA and sequence analysis of UF3GT genes

UF3GT genes of 'Tsuda' and 'Yurugi Akamaru' turnips were cloned according to the procedure described by Xu et al. (2008a). Bioinformatics analysis for sequenced nucleotide sequences was carried out using the software of basic local alignment sirBLAST alignment, open reading frame (ORF) search, molecular phylogenetic tree drawing and protein property prediction.

Northern blotting and prokaryotic expression

The probe was labeled with digoxigenin and the expression changes of UF3GT genes in the two kinds of turnips were detected through northern blotting, and repeated three times.

Nucleotide sequences containing Nde I and Not I restriction enzyme cleavage sites and protective bases were added to the both sides of target genes through two PCR reactions. The purification products of PCR and vector pET-14b were digested with Nde I and Not I restriction endonucleases after PCR purification products were recovered, and then the digested target fragments was recovered. With T4 DNA ligase, the digested target fragment of the PCR products and the vector were ligated together. Subsequently, the ligated products were transformed into competent cells of *Escherichia coli* DH5α through heat-shocking. After extracting plasmids, positive clones were screened by PCR and restriction digestion, and then verified by sequencing. The plasmids sequenced correctly were transformed into E. coli BL21 and verified by PCR reaction. The transformants containing recombinant plasmids pET-14b-BrUF3GT1 and pET-14b-BrUF3GT2 were inoculated into liquid Luria Bertani (LB) medium (containing ampicillin with the concentration of 50 mg l-1) and allowed to grow at 37°C. Before induction, 0.5 ml cultured bacteria was taken out as the samples when OD600 reached 0.5 to 0.6. Then, isopropylthio-β-galactoside (IPTG) was added into the remaining cultured bacteria to final concentration of 1 mmol l-1, and cultivation was continued to induce the expression of the target protein. After 3 h, 500 μl samples after induction together with the samples before induction were harvested by centrifugation at 12000 rpm for 1 min. After discarding the supernatant, the pellet was resuspended in 60 μl 1× sodium dodecyl sulphate (SDS) buffer solution. The suspended matter was heated at 100°C for 5 min and then centrifuged at room temperature for 1 min. Finally, SDS-polyacrylamide gel electrophoresis (PAGE) electrophoresis was performed using the supernatant, followed by analysis of the results of the induced protein. BrUF3GT1 and BrUF3GT2 fusion protein were purified using His-tag affinity column (HisTrapTMHP, GE Healthcare) after induced expression (Li et al., 2010b), and then the purified result was detected through SDS-PAGE electrophoresis.

RESULTS AND ANALYSIS

Cloning the full-length cDNA sequence of UF3GTs in 'Tsuda' and 'Yurugi Akamaru' turnips

For RT-PCR, total RNA from two kinds of turnips were extracted, and RT-PCR reaction was performed using gene-specific primers. As shown in Figure 1 (M is DNA molecular weight marker DL 2000), in 'Tsuda' and 'Yurugi Akamaru' turnips, amplified cDNA products were both about 1400 bp in agreement with the expectation. The recombinant plasmids were extracted after the experiment of purification, connection, transformation and expanding cultivation of RT-PCR products. Using the plasmids as template, identification PCR was performed with the gene-specific primers. Recombinant plasmids that could amplify the correct fragments were selected and subsequently sequenced.

Nucleotide sequence and deduced amino acid sequence analysis of UF3GTs in 'Tsuda' and 'Yurugi Akamaru' turnips

RT-PCR products of 'Tsuda' and 'Yurugi Akamaru'

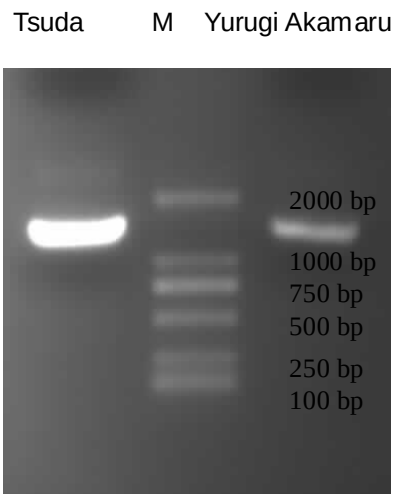

Figure 1. The RT-PCR product of *UF3GT* genes in 'Tsuda' and 'Yurugi Akamaru' turnips.

turnips were sequenced and proven to be UF3GT full-length cDNA sequence. In GenBank, the accession number of 'Tsuda' turnip UF3GT cDNA (BrUF3GT1) and 'Yurugi Akamaru' turnip UF3GT cDNA (BrUF3GT2) was JF 346162 and JF 346163, respectively, containing 1407 nucleotides. BLAST results showed that the sequence homology between BrUF3GT1 and UF3GT mRNA (XM_002865969) of *Arabidopsis lyrata* subsp. lyrata was 88%, and the sequence homology between BrUF3GT2 and UF3GT mRNA (NM_124785) of *A. thaliana* was 87%. BrUF3GT1 and BrUF3GT2 protein consisted of 468 amino acids, containing the complete ORF.

Homology alignment of amino acid sequence revealed that BrUF3GT1 and BrUF3GT2 protein were highly homologous to UF3GT protein from various plants. Of these plants, BrUF3GT1 and BrUF3GT2 shared 87% homology with both UF3GT (NP_200217) of *A. thaliana* and UF3GT (XP_002866015) of *A. lyrata* subsp. lyrata, and shared 61% homology with UF3GT of *Vitis vinifera* (XP_002282825). Phylogenetic tree analysis of UF3GT protein showed that BrUF3GT1 and BrUF3GT2 of turnip were evolutionarily closer to UF3GT of *A. thaliana*, but more distantly related to UF3GT of strawberry (*Fragaria ostanssa*) and mangosteen (*Garcinia mangostana*) .

The results of BlastP analysis of BrUF3GT1 and BrUF3GT2 showed that the protein peptide from 16th to 453th amino acids carried the common domain of glycosyltransferase family members, which could transfer activated glycosyl to substrate (Figure 2).

ProtParam software predicted that molecular weight of BrUF3GT1 and BrUF3GT2 was 51.8755 and 51.8896 kDa, respectively, theoretical isoelectric point was both 5.44. Valine accounted for the greatest proportion in amino acid composition, the percentage was both 9.4%. The coefficient instability of BrUF3GT1 and BrUF3GT2 protein was 41.03 and 41.19, belonging to unstable protein. The testing results from SignalP 3.0 Server suggested that BrUF3GT1 and BrUF3GT2 did not contain a signal peptide sequence and were not secreted proteins. Secondary structure prediction indicated that in the whole 468 amino acids sequence of BrUF3GT1 and BrUF3GT2, the amino acids that formed alpha helix accounted for 32.26 and 33.33%, random coil sequences accounted for 45.51 and 44.44% respectively, extended strand accounted for 22.22% both, and the two proteins did not contain other secondary structures. Prediction of Pfam HMM indicated that BrUF3GT1 and BrUF3GT2 belonged to the superfamily enzymes of UDP glycosyl transferase (UGT) and may catalyze transformation of glycosyl group of UTP-sugar molecules to hydrophobic molecules. ScanProsite analysis revealed that the amino acid sequence of BrUF3GT1 and BrUF3GT2 protein from 343 to 386 amino acids was WvqQpmILdhpsvgCFVSHCGfgSmweSLmsdcQMvlvPqhg EQ, corresponding to the tag sequence of glycosyltransferase family proteins, [FW]-x(2)-[QL]-x(2)-[LIVMYA]-[LIMV]-x(4,6)-[LVGAC]-[LVFYAHM]-[LIVMF]-[STAGCM]-[HNQ]-[STAGC]-G-x(2)-[STAG]-x(3)-[STAGL]-[LIVMFA]-x(4,5)-[PQR]-[LIVMTA]-x(3)-[PA]-x(2,3)-[DES]-[QEHNR], this motif was also included in the protein structure domain found by BlastP program. The alignment result using BioEdit software displayed that the BrUF3GT1 gene was 99% homologous to BrUF3GT2. These nucleotide sequences differed in only seven sites and the deduced amino acid sequence differed in only one site (Table 1).

UV-A induced expression of BrUF3GT1 and BrUF3GT2 genes

After treating 'Tsuda' and 'Yurugi Akamaru' turnips by UV-A for 0 and 48 h, the expression changes of BrUF3GT1 and BrUF3GT2 were detected. In the root tuber skin, without light, BrUF3GT1 and BrUF3GT2 genes showed low expression. After irradiation for 48 h by UV-A, the expression of BrUF3GT1 and BrUF3GT2 genes increased slightly (Figure 3).

In order to further study the relationship between the expression change of BrUF3GT1 and BrUF3GT2 genes and the exposure time irradiated by UV-A, we treated two kinds of turnips with different times. Northern blotting results showed that, in the root tuber skin of 'Tsuda' and 'Yurugi Akamaru' turnips without light, the expression of BrUF3GT1 and BrUF3GT2 genes was lower. After irradiating 'Tsuda' turnip for 18 h and 'Yurugi Akamaru' turnip for 12 h using UV-A, the expression of BrUF3GT1 and BrUF3GT2 genes increased significantly and reached the highest level (Figure 4). These findings indicate that the expression strength of BrUF3GT1 and BrUF3GT2 genes was correlated with the time irradiated by UV-A.

Table 1. Difference of nucleotide and amino acid sequence between *BrUF3GT1* and *BrUF3GT2*.

Nucleotide site	*BrUF3GT1* / *BrUF3GT2*	amino acid site	BrUF3GT1 / BrUF3GT2
381	A / T	168	Gly / Ala
503	G / C		
994	A / C		
1011	C / A		
1048	T / C		
1068	C / G		
1095	T / C		

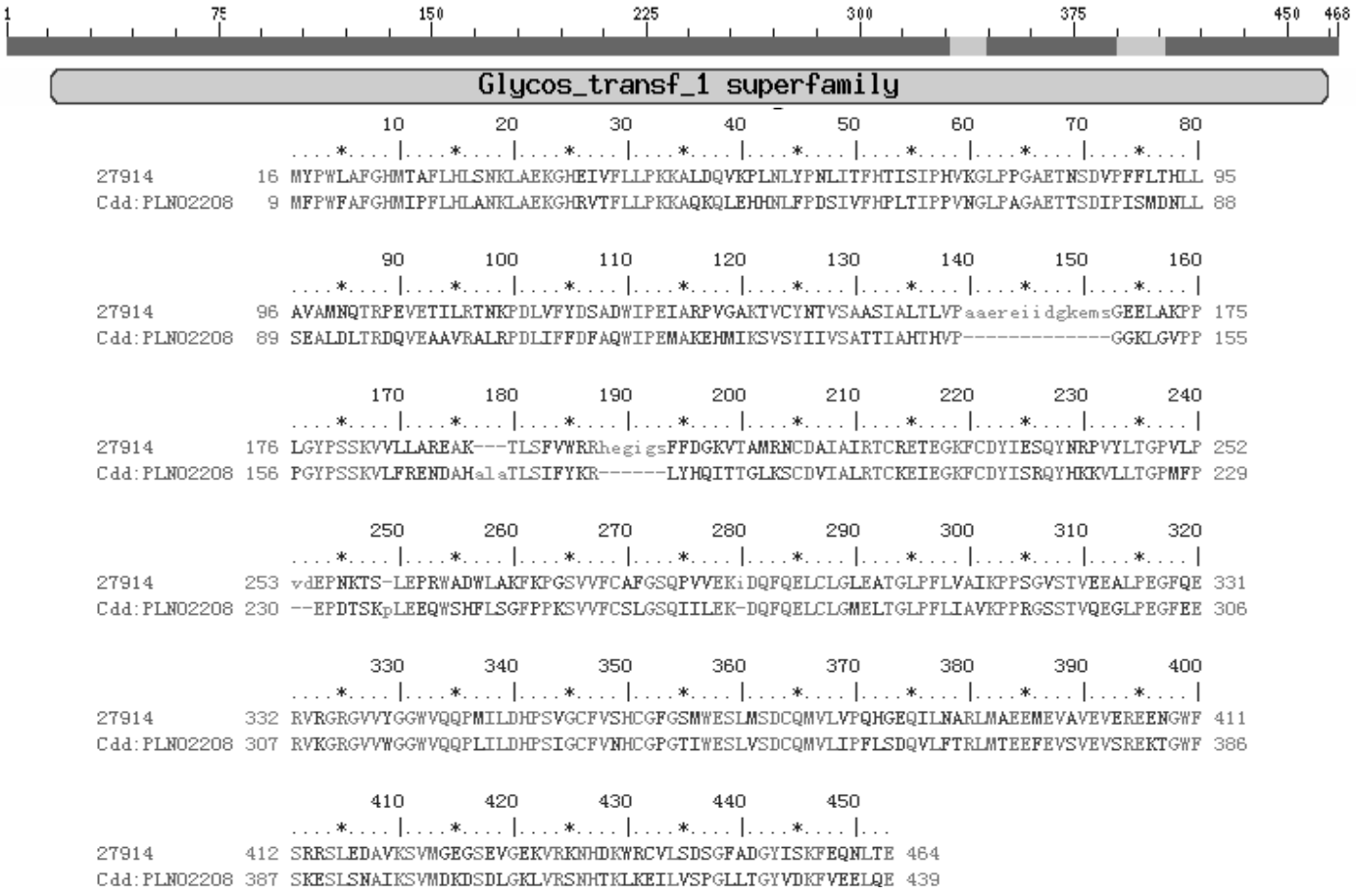

Figure 2. The domains of BrUF3GT1 and BrUF3GT2.

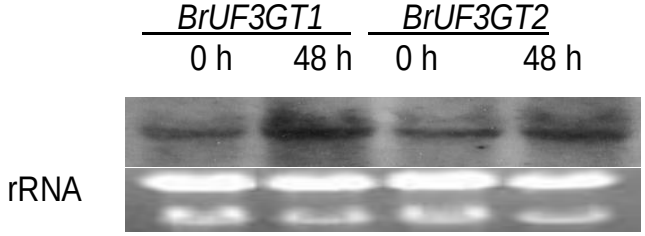

Figure 3. Expression of *BrUF3GT1* and *BrUF3GT2* genes after exposure to UV-A for 0 and 48 h.

Prokaryotic expression of BrUF3GT1 and BrUF3GT2 genes

After recovering and purifying the PCR products of BrUF3GT1 and BrUF3GT2 genes containing Nde I and Not I restriction sites, the purified products were digested with Nde I and Not I restriction endonucleases. The target fragments were recovered, and then ligated to the digested products of vector. After the transformation of ligated products, single colony was picked out to perform liquid expanding cultivation, and then the plasmid was

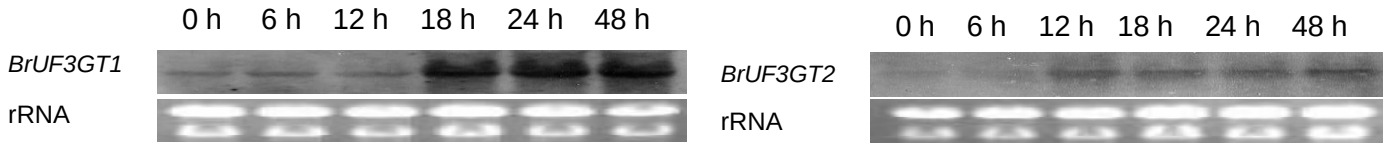

Figure 4. Expression of *BrUF3GT1* and *BrUF3GT2* genes after exposure to UV-A for different times.

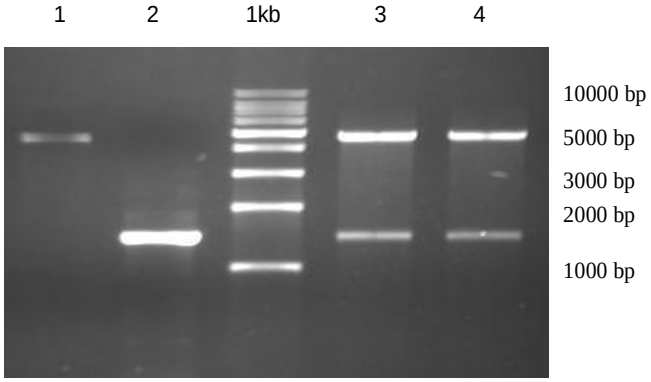

Figure 5. Enzyme digestion identification of pET-14b-BrUF3GT1and pET-14b-BrUF3GT2. 1, Empty vector digestion; 2, PCR positive control; 1 kb, marker; 3, pET-14b-BrUF3GT1 plasmid digestion; 4, pET-14b-BrUF3GT2 plasmid digestion.

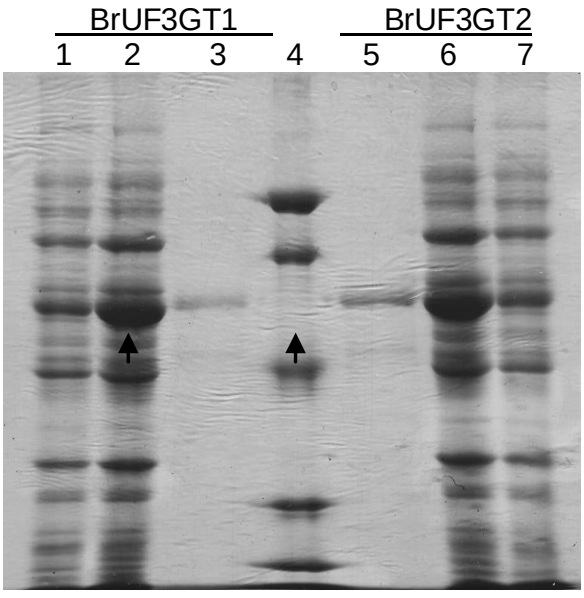

Figure 6. Purification of BrUF3GT1 and BrUF3GT2 fusion protein. 1, Before induction; 2, after induction; 3, purification; 4, protein marker; 5, purification; 6, after induction; 7, before induction.

extracted. The correct construction was verified with PCR and enzymes digestion test. The results of enzymes digestion is shown in Figure 5. After digestion, two bands of the pET-14b-BrUF3GT1 and pET-14b-BrUF3GT2 plasmids were produced, respectively. The band sizes corresponded to the enzymes digestion fragment of empty vector and the PCR products of BrUF3GT1 and BrUF3GT2, respectively. The results suggest that the prokaryotic expression vector was constructed successfully.

The recombinant plasmids were transformed into BL21. Following verification by PCR, the correct plasmids were selected to perform small amount of induction. Fusion protein was purified using His-tag protein purification kit produced by TOYOBO, followed by SDS-PAGE electrophoresis. The results indicated that the fusion protein formed inclusion bodies. Subsequently, the fusion protein was purified using HisTrapTMHP affinity column. The SDS-PAGE electrophoresis result showed that the fusion proteins of BrUF3GT1 and BrUF3GT2 had been purified successfully, and with the molecular weight of about 51.89 KDa. The arrow marks the purified target protein band (Figure 6).

DISCUSSION

UF3GT is not only an important catalytic enzyme of unstable anthocyanin glycosylation, but also a critical catalytic enzyme in the late stage of anthocyanin biosynthesis. UV and environmental stress can induce and regulate the biosynthesis of flavonoids such as plant anthocyanins (Guo et al., 2008; Päsold et al., 2010). Our study team also demonstrated that UV-A could induce anthocyanin accumulation and expression of catalytic enzyme genes in the root tuber skin of 'Tsuda' turnip (Zhou et al., 2007; Xu et al., 2008b). The UF3GT gene is a member of the glycosyltransferase family, existing downstream of each path of the anthocyanin biosynthetic pathway and stabilizing anthocyanin (Kovinich et al., 2010). Previous studies have indicated that *A. thaliana* UGT78D2 was associated with anthocyanin accumulation. In addition, the ectopic expression of UGT78D2 gene in *A. thaliana* mediated by the CAMV35S promoter could produce purple testa (Lee et al., 2005). The expression of UF3GT gene in grape was critical to anthocyanin biosynthesis. The UF3GT gene was only expressed in the red peel mutant (Kobayashi et al., 2001).

In this study, we cloned BrUF3GT1 and BrUF3GT2 genes of 'Tsuda' and 'Yurugi Akamaru' turnips. Homology

alignment result displayed that the deduced amino acid sequence of BrUF3GT1 and BrUF3GT2 protein was 87% homologous to the proteins of both *A. thaliana* and *A. lyrata* subsp. Lyrata. At the same time, BrUF3GT1 and BrUF3GT2 contained the domain of glycosyltransferase superfamily members, which plays an important role in the reaction of glycosyl transformation. This study establishes the foundation for separating the key catalytic enzyme genes of light-sensitive and light-insensitive anthocyanin biosynthesis and shows the mechanism of light-sensitive and light-insensitive anthocyanin biosynthesis.

The color formation of the root tubers in 'Tsuda' and 'Yurugi Akamaru' turnips indicates that the anthocyanin biosynthesis pathway in these two turnips is different in relationship with the light. Studying results in this paper show that UV-A could induce the expression of BrUF3GT1 and BrUF3GT2 genes, and the expression of these two genes was correlated with light-exposure time. Whether the BrUF3GT1 gene in 'Tsuda' turnip and the BrUF3GT2 gene in 'Yurugi Akamaru' turnip play an important role in the light-sensitive and light-insensitive anthocyanin biosynthesis, needs further experimental validation.

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