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Molecular cloning and tissue expression analyses of a UF3GT gene from Capsicum annuum L.

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In the present study, a novel gene designated as *UF3GT* was isolated from *Capsicum annuum* L. Sequence and structural analysis determined that the *UF3GT* protein, which contained 1401 base pairs encodes 447 amino acids and belongs to the Glycosyltransferase-GTB-type superfamily. The isoelectric point and the molecular weight of this gene are 5.97 and 49589, respectively. The deduced amino acid sequence shows highly identity with known anthocyanin synthases in other species. The phylogenetic tree analysis revealed that the *C. annuum* L. *UF3GT* has a closer genetic relationship with the *UF3GT* of petunia (*Petunia x hybrida*). The RT-PCR gene expression analysis indicated that the *UF3GT* gene was mostly expressed in pericarp, moderately expressed in stem, leaf, flower, placenta and seed, whereas no expression signal was detected in root. The study established the primary foundation for further research on this *C. annuum* L. gene.

Key words: Capsicum annuum L., semi-quantitative RT-PCR, glycosiltransferase gene, tissue expression profile analysis.

INTRODUCTION

Capsicum annuum L. (commonly known as hot pepper, chili, chili pepper, and bell pepper) is a dicotyledonous flowering plant. It belongs to family Solanaceae, which includes potato, tomato, eggplant, African eggplants etc. (Knapp, 2002; Hunziker, 2001). In China, people living in Yunnan province have planted this crop for thousands of years. The whole plant is violet black, especially the fruit. Shades ranging from violet to black pigmentation in *C. annuum* L. are attributed to anthocyanin accumulation.

Anthocyanins are plant pigments widely distributed in colored fruits and flowers. They also exhibit antioxidant activities and therefore may contribute to the prevention of heart disease, cancer, and inflammatory disease (Hou, 2003; Bagchi et al., 2004; Katsube et al., 2003). They are usually localized in the vacuoles of petal epidermal cells (Goto et al., 1982; Tanaka et al., 2004) and are responsible for diverse pigmentation from orange to red, purple and blue in flowers, fruits and vegetables. Often, these compounds also occur in leaves, stems, seeds, and other tissues. Contributing to the colorful appearance of flowers, fruits and vegetables, anthocyanins help them to attract animals, leading to seed dispersal and pollination (Harborne and Williams, 2000). Mazza and Miniati (1993) reported that anthocyanins might be important in protecting plants against ultraviolet-induced damage, as well. In addition, they play roles as anti-

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oxidants and in protecting DNA and the photosynthetic apparatus from high radiation fluxes. Recently, using the known genetic sequence to synthesize primers, several genes, such as *CHS*, *CHI*, *F3H*, *DFR*, *ANS*, etc have been cloned in grapes, apples, strawberries, and other plants (Jaakola et al., 2002; Li et al., 2001).

UF3GT (UDP glucose flavonoid 3-glucosyltransferase) has been shown to be one of the key enzymes in anthocyanins biosynthesis derived from the phenylpropanoid pathway. The UF3GTs play important roles not only in modifying flower color but also in increasing the solubility and stability of hydrophobic flavonoids (Hondo et al., 1992; Yoshida et al., 2000).

UF3GT have been isolated from flowers of many ornamental plants, including *Gentiana triflora* (Tanaka et al., 1996) and *Petunia x hybrida* (Yamazaki et al., 2002). They have also been isolated from *Zea mays* (Goto et al., 1982), *Antirrhinum majus* (Martin et al., 1991), *Vitis vinifera* (Ford et al., 1998), *Hordeum vulgare* (Wise et al., 1990), *Perilla frutescens* (Gong et al., 1997) and *Fragaria ananassa* (Almeida et al., 2007). Many *UF3GT* genes have been cloned and heterologously expressed, which makes in-depth study of the molecular mechanism controlling anthocyanin biosynthesis possible. Semiquantitative RT-PCR has been conducted to analyze the relative expression levels in various tissues. This study provides primary information for further understanding the biochemical functions of *UF3GT* in *C. annuum* L.

MATERIALS AND METHODS

Sample collection

All plants used in the study were derived from College of Horticulture, Yunnan Agricultural University. Yunnan Purple Pepper No.1 (*C. annuum* L.) tissues (root, stem, leaf, blossom, pericarp, placenta, and seed) were instantly frozen in liquid nitrogen and stored at -80°C before use.

Total RNA extraction and first-strand cDNA synthesis

Total RNA from Yunnan Purple Pepper No.1, was extracted by the Trizol procedure (TaKaRa) and cDNA was synthesized using High Fidelity PrimeScript RT-PCR Kit (TaKaRa) according to the manufacturer's protocol.

PCR amplification

The PCR was performed to isolate the Yunnan Purple Pepper No.1 gene using the pooled cDNAs from different tissues. The 20 µl reaction mixture contained 1.5 µl (25 ng/µl) DNA, 1 µl 2.5 mM mixed dNTPs, 2 µl 10 x Taq DNA polymerase buffer (MgCl₂ plus), 0.4 µl 10 µM forward and reverse primer, 0.3 µl 5 U/µl Taq DNA polymerase and 13.4 µl sterile water. The PCR program initially started with a 94°C/4 min, followed by 35 cycles of 94°C/1 min, 57°C/45 s, 72°C/90 s, and then 72°C extension for 10 min, finally 4°C to terminate the reaction. The mRNA and amino acid sequences for UF3GT genes from various plant species available in the databank of National Centre for Biotechnology Information

(http://www.ncbi.nlm.nih.gov) were used to locate conserved boxes by multiple sequence alignment through CLUSTALW 1.8 and primers with the following sequence were designed. UF3GT-F: ATGACTACTTCTCAACTTCATATT, UF3GT-R: TYAAGTARGCTTGTGACATTTAA (C/T= Y, A/G= R).

RT-PCR for expression profile

The primers of Yunnan Purple Pepper No.1 *UF3GT* gene which were used to perform the RT-PCR for tissue expression profile analysis was same discussed above. The 20 µl mixture contained was: 4 µl 5×PrimeSTAR PCR Buffer, 0.4 µl dNTP Mixture (10 mM each), 0.2 µl of forward and reverse primer (10 µM), 0.2 µl of PrimeSTAR HS DNA Polymerase (2.5 U/µl) (TaKaRa), 2 µl aliquot of cDNA and 13 µl RNase Free dH₂O water. The products of amplification were checked on a 1.5% agarose gel and visualized with ethidium bromide.

Bioinformatics analysis

PCR amplification was repeated 5 times independently. The products were cloned into pMD18-T vector (TaKaRa) and sequenced bidirectionally. At least 10 independent clones were sequenced for each PCR product. Sequencing data were edited and aligned using DNASTAR software (DNAStar Inc., Madison, Wisc.). The cDNA sequence was predicted using the GenScan software (http://genes.mit.edu/GENSCAN.html). Putative protein theoretical molecular weight (Mw) and isoelectric point (pl) prediction, signal peptide prediction, subcellular localization prediction and transmembrane topology prediction were performed using the Compute pl/Mw Tool (http://us.expasy.org/tools/pi_tool.html), SignalP 3.0 server (http://www.cbs.dtu.dk/services/SignalP/), PSort Ш (http://psort.hgc.jp/), TMpred

(http://www.ch.embnet.org/software/TMPRED_form.html), respectively. The Blastp program and Conserved Domain Architecture Retrieval Tool were used to search for similar proteins and conserved domain, respectively (http://www.ncbi.nlm.nih.gov/Blast). The alignment of the nucleotide sequences and deduced amino acid sequences were computed using ClusterX, and the phylogenetic trees were computed using the ClustalX and Mega 4.0 softwares with standard parameters. Secondary structures of deduced amino acid sequences were predicted with SOPMA (http://npsa-pbil.ibcp.fr/). The 3D structures were predicted based on the existed 3D structures by the amino acids homology modeling on swiss server (http://swissmodel.expasy.org/).

RESULTS

RT-PCR results for *C. annuum* L. *UF3GT* gene

Through RT-PCR with pooled tissue cDNAs, for *C. annuum* L. *UF3GT* gene, the resulting PCR products were about 1430 bp long (Figure 1).

cDNA nucleotide sequence analysis using the BLAST software at NCBI server (http://www.ncbi.nlm.nih. gov/BLAST) revealed that this gene was not homologous to any of the known *C. annuum* L. genes .The sequence prediction was carried out using the GenScan software and the results showed that the cDNA sequences encoded 447 amino acids.

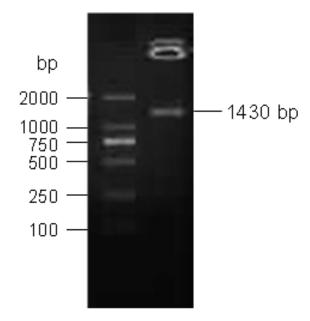


Figure 1. Analysis of PCR products on 1.5% agarose gels.

ATGACTACTTCTCAACTTCATATTGCAGTTCTTGCTTTTCCTTTTGGTAGCCATGCAGTTCCTTTACTCACACTTGTCCAAAAAAATCTCT M T T S Q L H I A U L A F P F G S H A U P L L T L U Q K I CCATTTTTACCATCAAACTCAAATTTTTTCCTTCCTTCAACACATCAAAATTCCCAACACCTCAATTTTCTCAAAAAGCTCCAAAACCAAGAAAAC L P S N S I F S F F N T S N S N T S I F S K A P N O E N ATCAAGATTTACAATGTTTGGGATGGTGTCAAACAAGGCAATGACACCCCTATTGGTTGTGAAGCCATACAAGTCTTCATACAATCAACT I K I Y N U W D G U K Q G N D T P I G C E A I Q U F I Q S T CCAACTAATTTCGAAAAATCAATGAAAGAGGCAGGAGGAAAATAGGTGTGAAGTTTTCTTGCATTATCAGTGATGCATTTTTATGGTTT P T N F E K S M K E A E E E I G U K F S C I I S D A F L W F C D F A K K M N U P W I A F W T A G S C S L S U H L TTAATTCGATCGAACGAGGAAACATTATTAAAAATTCCAGGATTTTCAACAAATTTAAGGATCAGTGACATGCCACAAGAAGTTGTAGCA LIRSNEETLLKIPGFSTNLRISDMPOEUUA CATAATTTAGAGGGGGGGATTTCCATCTTTGTTATACAACATGGCACTAAATTTGCACAAAGCAGATGGTGTTGTACTGAACTCTTTCGAG H N L E G G F P S L L Y N M A L N L H K A D G V V L N S F E GAATTGGATCCAAAAAATCAACAATGATCTCAAAATCAAAGCTCCCAAAAGGTACTAAATATTGGCCCTTTAGTCCTACAGTCATCAAAGAAA E L D P K I N N D L K S K L Q K U L N I G P L U L Q S GTTATTTCAGATGTTAATTCTGATGAAAGTGGATGTATCAAATGGCTTGACAAGCAAAATGAAAAATCAGTTGTGTACTTAAGTTTTGGT U I S D U N S D E S G C I K W L D K O N E K S U U Y L S F ACTGTCACAAACATTACCTCCTAATGAAATTGTGGCAATAGCAGAAGCATTAGAAGCCAAAAGGGTACCTTTTATTTGGTCATTAAAAGAC L P P N E I V A I A E A L E A K R V P F AATGGGGTCAAGATTTTGCCTAAAGGTTTTCTTGAAAGGACAAATGAATTTGGGAAAATAGTTTCTTGGGCACCCCAATTGGAAATCTTG N G U K I L P K G F L E R T N E F G K I U S W A P O L E I L A H S S U G U F U T H C G W N S I L E S I S Y G U P M I C R CCTTCTTTTGGTGACCAAAAGTTGAATAGTAGAATGGTGGAGAATGTTTGGCAAATTGGTTTGCAAATTGAAGGTGGGAATTTCACTAAA PSFGDOKLNSRMUENUWOIGLOIEGGNF AGTGGAACAATTAGTGCATTGGATACTTTTTGCAATGAGGATAAAGGTAAGGTATTAAGGCAAAATGTTGAAGGGCTAAAAGAAAAAGAAAAGCA S G T I S A L D T F C N E D K G K V L R Q N V E G L K E K A TTAGAAGCTGTGAAACCAAATGGGAGTTCAACAGAAAATTTCAAGGTTCTAGTTGAGCTAATTAAATGTCACACCCCtACTTGA L E A U K P N G S S T E N F K U L U E L I K C H T P Т

Figure 2. The complete coding sequence of *Capsicum annuum* L. *UF3GT* gene and its encoding amino acids * indicates the stop codon.

The theoretical pl and Mw of this deduced protein of this *C. annuum* L. gene was computed using the Compute pl/Mw Tool. The pl of this gene was 5.97 while molecular weight of the putative proteins was 49,589.

Prediction of protein properties

The complete coding sequence (CDS) of this gene and the encoded amino acids are presented in Figure 2.

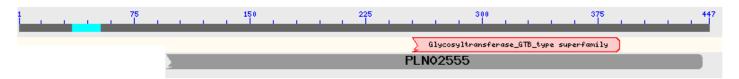


Figure 3. The conservative domains of the protein encoded Capsicum annuum L. UF3GT gene.

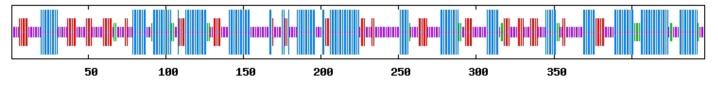


Figure 4. Secondary structure of UF3GT protein in Capsicum annuum L. Helices, strands and coils are indicated, respectively, with long, middle and short vertical lines.

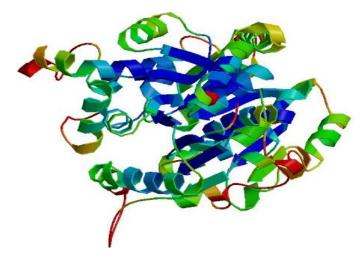


Figure 5. The tertiary structure of UF3GT.

The putative protein was also analyzed using the Conserved Domain Architecture Retrieval Tool of Blast at the NCBI server (http://www.ncbi.nlm.nih.gov/BLAST) and the conserved domain was identified as PLNO2555 domain (Figure 3). According to Figure 3, we can see it belongs to Glycosyltransferase-GTB-type superfamily.

The secondary structure analysis of the deduced amino acid sequence by GOR algorithm indicated that the protein consisted of 40.94% a-helix, 37.36% random coils and 17.00% β-sheets (Figure 4). The signal peptide prediction performed by SignalP 3.0 on the basis of a combination of several artificial neural networks and hidden Markov models revealed that C. annuum L. UF3GT contained potential signal peptide with 99.9% probability (Bendtsen et al., 2004). Transmembrane TMpred prediction made topology by (http://www.ch.embnet.org/software/TMPRED form.html) , showed that C. annuum L. UF3GT had N-terminus outside with 4 strong transmembrane helices. For subcellular localization analysis, the amino acid sequence was submitted to the PSORT program, and Reinhardt's method showed *UF3GT* was probably located in the cytoplasm with up to 76.7% probability (Nakai and Horton, 1999).

Homology modeling

In order to better understand the detailed structures of UF3GT, the homology modeling was performed to estimate its 3D structure. The 3D structure of UF3GT (2-443AA) by homology modeling was based on template-3hbjA (chain A) with 43.1% sequence identity (Guex and Peitsch, 1997; Schwede et al., 2003; Arnold et al., 2006). The 3D structure analysis may provide a basis for further studying the relationship between structure and function of UF3GT (Figure 5).

Capsicum annuum Prunus avium Vitis lahrusca Arabidopsis thaliana Petunia x hybrida Dianthus caryophyllus	-MTTSQLHIAU LAFPFGSHAU PLLTLUQKIS PFLPSNSIFS FFNTSNSNTS IFSKAPN QENIKIYNUW DGUKQGN-DT PI APQPIDDD HUUVEH.U.ASTS .T.A.IRRLA AAS.N-TLSQN. LN-TNTNL PRUFD.APD.YUFA GK SQTTTNP
Capsicum annuum Prunus avium Vitis lahrusca Arabidopsis thaliana Petunia x hybrida Dianthus caryophyllus	GCEAIQUFIQ STPTNFEKSM KEAEEEIGUK FSCIISDAFL W-FCCDFAKK MNUPWIAFWT AGSCSLSUHL YTDLIRSN EETLLKI PQ.D.EL.MK AA.HTT.L DACUAHT.KR LT.L.T
Capsicum annuum Prunus avium Vitis lahrusca Arabidopsis thaliana Petunia x hybrida Dianthus caryophyllus	PGFSTHLRIS DMPQEUUAHH LEGGFPSLLY HMALHLHKAD GUULNSFEEL DPKIHHDL KSKLQ-KULH IGPLUL-QSS KKUIS DUHSDESGCI KW MKUK .L.EG.IFGDSU.SRM.H Q.GQL.PR.H A.LUITUTFM-LL. UFH.AAAA SPPLPEALTA ADDUTL S. MY-EU.FR .LQEGI.FGNSL.SRM.H R.GQU.PT A.FIDSLTK-TYFH.ITPP PUPH TTL Q. S.ME-KI.UK .TQEG.FGDSU.SKT.H Q.G.A.PR.T A.FITFT.F R.EFK-RYA.LS.P SQT. TLUH.PH.LL A. ST.KPT SPKKU LDAG.R.P.T ST.KPT SPKKU LDAG.R.R.I LU L.MGL.SGD .DAP.SQM.H K.GTM.PK U.TU.Q. SLUU MK-L.C UFP.TCPP QUY.PDH.L D.
Capsicum annuum Prunus avium Vitis lahrusca Arabidopsis thaliana Petunia x hybrida Dianthus caryophyllus	LDKQNEKS UVYLSFGTUT TLPPNEIVAI AEALEAKRUP FIWSLKDNGU KILPKGFLER TNEFGKI USWAPQLEIL AHSSUGUFUT HCGWNSILES IS K-AASSUS.A RP-EK.LM.M .QSG. LSFK TP.LNEL.UK ASM.MU .PPRUAAS.L.T .A .KERK-PTSI P.A.L.LSR.KAR UH.EK .RGY.MU .PA.UEAALW. UA IE.RSTAS .A.IAR.A .P.U.LQG.SSKUQUMM TH.EDRQ.MU .PU.L .H.EAMS .GUU. .E.K.E
Capsicum annuum Prunus avium Vitis lahrusca Arabidopsis thaliana Petunia x hybrida Dianthus caryophyllus	YGUPMICRPS FGDQKLMSRM UENUWQIGLQ IEGG-HFTKS GTISALDTFC NEDKGKULRQ NUEGLKEKAL EAUKP-NGSS TEMFKULUEL IKCHTPT- GFX.RU.A.TD.LEUT U.DUHL.KYF.QUL SQQRKM.G.INTU.LL.Q QS.EK AQL.LDU .SGS.KU- GLFRGD.LEURU I.M.CF.QIL SQCKE .LRA.R.T.D RGKT.D. USKPKDU- AIHAI.A.SAE.UT .SSUD .FEES.RUL UQ.DKMKU .AKK.E.L.QST-K FGG.LDE UUNFG CFFSKD.U.U.KL TL.I IE.Q.I .IELKF.IGC.QTMHQUL ASE.EEM.KE.KL.E .S.Q-G NNA.ITSS

Figure 6. The alignment of the protein encoded by C. annuum L. UF3GT with UF3GT from other species.

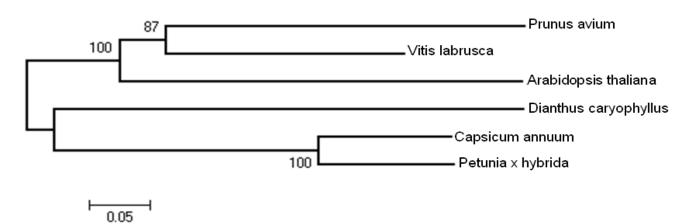


Figure 7. Phylogenetic tree analysis for selected UF3GT protein.

Sequence analysis and evolutionary relationships

Further BLAST analysis of this protein revealed that *C.* annuum L. UF3GT has high homology with the anthocyanin synthases protein UF3GT of 5 species -Prunus avium, V. labrusca, Arabidopsis thaliana, Dianthus caryophyllus, P. × hybrida (Figure 6).

Based on the result of the alignment of *UF3GT*, the phylogenetic tree was constructed using the ClustalW software (http://www.ebi.ac.uk/clustalw), as shown in Figure 7. The phylogenetic tree analysis revealed that the *C. annuum* L. *UF3GT* has a closer genetic relationship with the *UF3GT* of petunia.

Gene expression profile analysis was carried out and results revealed that the *C. annuum* L. *UF3GT* gene was highly expressed in pericarp, moderately expressed in stem, leaf, flower, pericarp, placenta and seed. There was no expression of *C. annuum* L. *UF3GT* in root (Figure 8).From the tissue expression profile analysis in our experiment it can be seen that this gene was obviously differentially expressed in some tissues and there were no expression in some tissues. As we did not study functions at protein levels yet, there might be many possible reasons for differential expression of this gene. The suitable explanation for this under current conditions is that at the same time those biological activities related

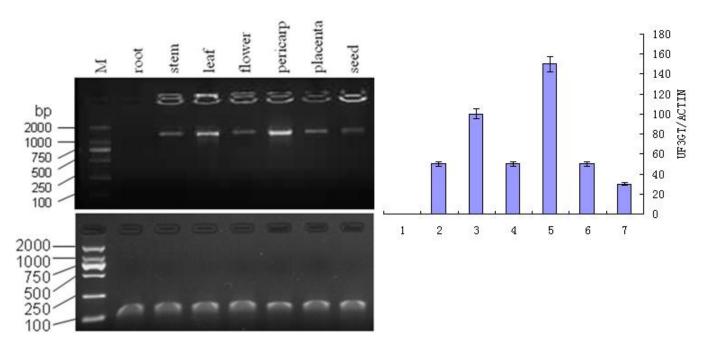


Figure 8. Tissue expression distribution of C. annuum L. UF3GT gene. M, DL2000 markers.

to anthocyanosides synthesis of this gene were presented diversely in different tissues.

DISCUSSION

UF3GT gene have been cloned and characterized from horticulture crops, such as Petunia (Weiss et al., 1995; Moalem-Beno et al., 1997), Periwinkle (Ohlsson and Berglund, 2001), Rose (Zieslin et al., 1974), and a few other species. In this study, a 1430-bp full-length cDNA gene UF3GT was isolated from C. annuum L.. The deduced amino acid sequence of UF3GT showed extensive similarity to their counterparts in other species. Through tissue expression analyses, we detected UF3GT expression in stem, leaf, flower, pericarp, placenta and seed where anthocyanin was concentrated, the expression of UF3GT was higher in pericarp. This phenomenon is in accordance with the anthocyanin content in these tissues (data not given). These data suggest that UF3GT maybe a specific tissue expression gene. And it was consistent with previous research (Hughes and Hughes, 1994). Anthocyanin content might have close correlation with UF3GT expression levels in response to tissue specificity and genetic background.

In this report, the complete coding sequences of the *C.* annuum L. UF3GT gene was isolated based on the conserved sequence information of the *P. xhybrida* and some referenced *C. annuum* L. ESTs. Sequence identification further validated that comparative genomics method is one useful tool to isolate the unknown genes especially the conserve coding region of genes for *C.* annuum L. or other plants. In conclusion, we have isolated the *C. annuum* L. *UF3GT* gene and performed necessary functional analysis and tissue expression profile analysis. This established the primary foundation for further research on *C. annuum* L. *UF3GT* gene.

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