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Molecular Characterization and Expression Pattern of a Novel Flavonoid 3'-Hydroxylase Gene

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Abstract: Flavonoids have significant health benefits for animals and humans. Flavonoid 3'-hydroxylase gene have been characterized to be required for flavonoid biosynthesis. In present experiment, the complete mRNA sequence of tobacco flavonoid 3'-hydroxylase gene was amplified using the rapid amplification of cDNA ends methods. The full-length tobacco flavonoid 3'-hydroxylase gene mRNA was 1828 bp containing an 1578 bp open reading frame which encodes a protein of 515 amino acids. Sequence analysis revealed that the flavonoid 3'-hydroxylase genes are conserved in plants. Results also showed that tobacco flavonoid 3'-hydroxylase gene has a closer genetic relationship with the flavonoid 3'-hydroxylase gene of *Lycopersicon esculentum*. Prediction of transmembrane helices showed that tobacco flavonoid 3'-hydroxylase might be a transmembrane protein. The expression profile was studied and the results indicated that tobacco flavonoid 3'-hydroxylase gene was highly expressed in in leaf and flower. These results established the primary foundation of utilization of tobacco flavonoids in the future.

Key words: Flavonoid, tobacco, flavonoid 3'-hydroxylase, expression pattern, flower

INTRODUCTION

In recent years, researchers including nutritionists and veterinarians had payed more attention to flavonoids for their desirable biological functions to humans and animals. Flavonoids are a major class of plant secondary metabolites including chalcones, flavanones, dihyroflavonols, flavans, anthocyanins, flavones, flavonols and isoflavonoids (Winkel-Shirley, 2001). Flavonoids are commonly found in plants and have significant health benefits for animals and humans. Many studies have proven the beneficial effects of flavonoids in atherosclerosis progression and cardiovascular disease. Dietary flavonoids reduce oxidative stress and exert anti-inflammatory actions. Moreover, flavonoids have the ability to avoid the thrombus formation, improve endothelial function, modify lipid levels and regulate glucose metabolism (Toh *et al*., 2013; Liu *et al*., 2013; Siasos *et al*., 2013). Several important genes including flavonoid 3'-hydroxylase gene have been characterized to be required for flavonoid biosynthesis. Flavonoid 3'-hydroxylase is a cytochrome P450-dependent mono-oxygenase and has an influence on the hydroxylation pattern which is an important structural feature in determining the color and stability of flavonoid compounds. It catalyzes the hydroxylation of both naringenin to eriodictyol and dihydrokaempferol to dihydroquercetin (Brugliera *et al*., 1999; Sharma *et al*., 2012).

Flavonoid 3'-hydroxylase genes have been isolated from many plants such as soybean, tomato and apple. Until today, the tobacco flavonoid 3'-hydroxylase gene has not been reported yet. In present experiment, researchers will isolate the complete mRNA sequences of this tobacco gene, subsequently perform some necessary sequence analysis and tissue expression analysis for this gene. These will establish the primary foundation of utilization of tobacco flavonoids in the future.

MATERIALS AND METHODS

Samples collection, RNA extraction and first-strand cDNA synthesis: Tobacco plants (Chinese local variety Yunyan 85) were grown in a naturally lit glasshouse with normal irrigation and fertilization. The tissues including leave, stem, root, flower were harvested and immediately frozen in liquid nitrogen and stored at -80°C. Total RNA extraction and first-strand cDNA synthesis for these tissue samples were performed as the methods describe by Liu (2009).

5' and 3'-RACE: The 5' and 3'-RACE were performed as the instructions of SMART™ RACE cDNA Amplification kit. For the tobacco flavonoid 3'-hydroxylase gene, the Gene Specific Primers (GSPs) were designed based on one tobacco EST sequence: FG135317. 5'-RACE GSP: 5'-CTGTGCTTGAGGACGTGTCTGTTCC-3'3'-RACEGSP: 5'-GGCGATAATGAAGGAGGAAAGCTCA-3'.

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Table 1: qRT-PCR primers for tobacco flavonoid 3'-hydroxylase, actin genes and annealing temperature

Gene	Primer sequence	Ta (°C)	Length (bp)
Flavonoid 3'-hydroxylase	Forward: 5'-GTTACCCGTTGACACTACCA-3'	58	305
	Reverse: 5'-CCTAAGCATACGCCACCT-3'		
Actin	Forward: 5'-CCATTCTTCGTTTGGACCTT-3'	56	257
	Reverse: 5'-TTCTGGGCAACGGAACCT-3'		

RACE touchdown PCRs were carried out with 5 cycles of 94°C 30 sec and 72°C 3 min followed by 5 cycles of 94°C 30 sec, 67°C 30 sec and 72°C 3 min, finally with 25 cycles of 94°C 30 sec, 67°C 30 sec, 72°C 3 min to terminate reaction. These RACE PCR products were then cloned into PMD18-T vector (TaKaRa, China) and sequenced bidirectionally with the Commercial Fluorometric Method.

Quantitative Real Time PCR (qRT-PCR) for tissue expression profile analysis: qRT-PCR for evaluating the level of mRNA for flavonoid 3'-hydroxylase gene was performed by the ABI Prism 7300 Sequence Detection Systems (Applied Biosystems, Foster city, CA, USA). The 25 μL reaction volume of PCR reaction contained 1 μL SYBR Green real-time PCR Master Mix, 100 ng cDNA template and 200 nM each primer. Conditions for real-time PCR were: an initial denaturation at 95°C for 3 min, 40 cycles of 95°C for 15 sec, optimal annealing temperature for each specific primer for 15 sec (Table 1), 72°C for 20 sec. The gene relative expression levels were quantified relative to the expression of the reference gene, actin (GenBank Accession No. GQ339768) by employing the $2^{-\Delta\Delta C}$T Value Model (Livak and Schmittgen, 2001).

Sequence analysis: The gene prediction of cDNA sequence was performed by GenScan Software (http://genes.mit.edu/GENSCAN.html). The protein analysis were performed using the BLAST tool at the National Center for Biotechnology Information (NCBI) server (http://www.ncbi.nlm.nih.gov/BLAST) and the Clustalw Software (http://www.ebi.ac.uk/clustalw).

RESULTS AND DISCUSSION

RACE results for tobacco flavonoid 3'-hydroxylase gene: For tobacco flavonoid 3'-hydroxylase gene through 5'-RACE, one PCR product of 967 bp was obtained. The 3'-RACE product was 955 bp. These products were then cloned to T-vector and sequenced. Taken together, a 1828 bp cDNA complete sequence was finally obtained (Fig. 1).

Sequence analysis: BLAST analysis of this cDNA nucleotide sequence revealed that this gene was not homologous to any of the known tobacco gene and it was then deposited into the Genbank database (Accession number: KF701483).

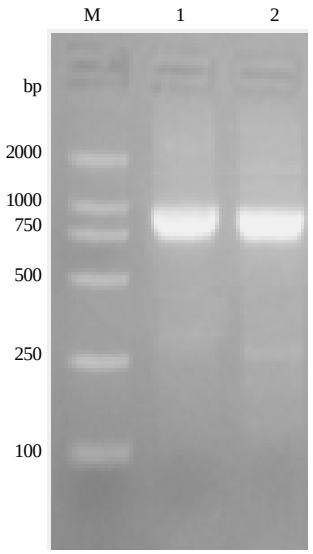

Fig. 1: RACE results for tobacco flavonoid 3'-hydroxylase gene. M: DL2000 DNA markers; 1: 5'-RACE product for tobacco flavonoid 3'-hydroxylase gene; 2: 3'-RACE product for tobacco flavonoid 3'-hydroxylase gene

The sequence prediction was carried out using the GenScan Software and results showed that the 1828 bp cDNA sequence represents one single gene which encodes 515 amino acids (Fig. 2). The theoretical isoelectric point (pI) and Molecular weight (Mw) of the deduced proteins of these three tobacco genes were also computed using the Compute pI/Mw tool (http://www.expasy.org/tools/pi_tool.html). The pI of tobacco flavonoid 3'-hydroxylase is 7.26. The molecular weight of this putative protein is 57123.19.

Further, BLAST analysis of this protein revealed that tobacco flavonoid 3'-hydroxylase shares high homology with the flavonoid 3'-hydroxylase of *Lycopersicon esculentum* (Accession number: XP_004236007, 86%), *Camellia nitidissima* (Accession number: ADZ28515, 76%), cacao (Accession number: EOY22049, 75%), *Cosmos sulphureus* (Accession number: ACO35752, 75%), wine grape (Accession number: BAE47005, 74%), *Vitis amurensis* (Accession number: ACN38268, 74%), *Bidens aurea* (Accession number: ACO35753, 74%), *Camellia sinensis* (Accession number: ACV74415, 74%), *Hieracium pilosella* (Accession number: ABC47161, 74%), chicory (Accession number: ACN65825, 73%),

African marigold (Accession number: ACO35756, 73%), *Dahlia pinnata* (Accession number: ACO35754, 73%), apple (Accession number: ACR14867, 73%), *Gynura bicolor* (Accession number: BAJ17668, 73%), Chinese peony (Accession number: AFI71898, 73%), *Rudbeckia hirta* (Accession number: ACO35757, 73%), *Eupatorium adenophorum* (Accession number: ABM46853, 72%), osteospermum hybrid cultivar (Accession number: ABB29899, 72%), *Chromolaena odorata* (Accession number: AEA06595, 72%), tree peony (Accession number: AEN71546, 72%), gerbera hybrid cultivar (Accession number: ABA64468, 72%) and sweet cherry (Accession number: ADZ54783, 71%). Its conserved domains was identified as P450 superfamily (Fig. 3).

The prediction of transmembrane helices in protein using the TMHMM Server V. 2.0 (http://www.cbs.dtu.dk/services/TMHMM/) showed that tobacco flavonoid 3'-hydroxylase protein might be a transmembrane protein (Fig. 4).

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CCGGATTCCCGGGATCACCAAATGGCAATCTTTTCCCTAATTCTCTACACTGTCATTTTCTCTTTTCTTCTACATTCCATTTTCAGCTTA
                  M  A  I  F  S  L  I  L  Y  T  V  I  F  S  F  L  L  H  S  I  F  S  L
TTTTTCCGCAAACGTTACCCGTTGACACTACCACCGGGTCCAAAACCATGGCCAATAATCGGAAACCTAGTCCATATGGGTCCAAAGCCG
 F  F  R  K  R  Y  P  L  T  L  P  P  G  P  K  P  W  P  I  I  G  N  L  V  H  M  G  P  K  P
CACCAATCAACTGCAGCCATGGCTCGAACCTACGGTCCACTCATGCACCTTAAGATGGGGTTCGTGGACGTGGTGGTTGTGGCGTCTGCG
 H  Q  S  T  A  A  M  A  R  T  Y  G  P  L  M  H  L  K  M  G  F  V  D  V  V  V  V  A  S  A
TCGGTGGCGGCTCAGTTCTTAAAAACTCATGACGCTAACTTCTCGAGCCGCCCTCCGAACTCGGGTGCAAAACACTTGGCTTACAATTAT
 S  V  A  A  Q  F  L  K  T  H  D  A  N  F  S  S  R  P  P  N  S  G  A  K  H  L  A  Y  N  Y
CAGGATCTTGTTTTTGCACCCTACGGACCAAGGTGGCGTATGCTTAGGAAAATTTGCTCTGTTCATCTTTTCTCTGCCAAGGCTTTAGAT
 Q  D  L  V  F  A  P  Y  G  P  R  W  R  M  L  R  K  I  C  S  V  H  L  F  S  A  K  A  L  D
GACTTCAGCCATGTCCGCCAGGATGAAGTAAGAACACTTACGCGCGCCCTAGCAAGTGCTGCGCAAAAACCGGTCAAGTTAGGCCAACTG
 D  F  S  H  V  R  Q  D  E  V  R  T  L  T  R  A  L  A  S  A  A  Q  K  P  V  K  L  G  Q  L
TTGAACGTGTGCACCACGAATGCACTTGCGCAAGTGATGCTAGGGAGGCGGGTGTTTGCTGACGCAAATGGCGGTGTTGATCCACAGGCG
 L  N  V  C  T  T  N  A  L  A  Q  V  M  L  G  R  R  V  F  A  D  A  N  G  G  V  D  P  Q  A
GAAGAGTTCAAGTCAATGGTGGTGGAGGCAATGGTGCTCGCCGGCGTTTTCAACGTTGGCGATTTTATTCCGGCACTCGATTGGTTGGAC
 E  E  F  K  S  M  V  V  E  A  M  V  L  A  G  V  F  N  V  G  D  F  I  P  A  L  D  W  L  D
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 I  Q  G  V  A  A  K  M  K  K  L  H  A  R  F  D  A  F  L  T  S  I  L  E  E  H  K  S  N  Q
TTTGGAGAAACGAAAGAACATGAGGACTTGTTGAGTACGTTAATCTCTTTGAAAAAAGAAGAAGGCGATAATGAAGGAGGAAAGCTCACA
 F  G  E  T  K  E  H  E  D  L  L  S  T  L  I  S  L  K  K  E  E  G  D  N  E  G  G  K  L  T
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 C  E  I  N  G  Y  F  I  P  K  G  S  T  L  L  V  N  V  W  A  I  A  R  D  P  N  E  W  A  D
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 P  L  E  F  R  P  E  R  F  L  P  G  G  E  K  P  K  V  D  V  R  G  N  D  F  E  V  I  P  F
GGAGCTGGGCGTAGAATCTGTGCTGGTATGAATTCGGGCATACGAATGGTCCAGTTGATGACTGCAACTTTAATTCATGCATTTAACTGG
 G  A  G  R  R  I  C  A  G  M  N  S  G  I  R  M  V  Q  L  M  T  A  T  L  I  H  A  F  N  W
GATTTGTCCATTGGACAATCGCCTGAGAAACTAAACATGGAGGAAGCATTTGGGCTGACTTTGCAACGGGCTGATCCATTGGTGGTGCAC
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 P  C  L  R  L  E  A  Q  A  Y  I  G  *
CCCAATTAGGATATGACATGTCCGCTAAATGTGTTAGTGTCTATTCGTTTATGCTTTCAAACGGAGGGAGTAGTAGTTTGTATTGAAAAATT
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TTTAAAAAAAAAAAAAAAAAAAA
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Fig. 2: The complete mRNA of tobacco flavonoid 3'-hydroxylase gene and its encoding amino acids. *Indicates the stop codon

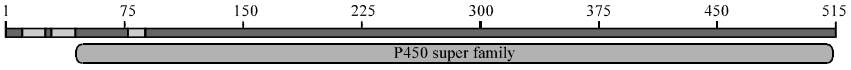

Fig. 3: The putative P450 superfamily domain of the protein encoded by tobacco flavonoid 3'-hydroxylase gene

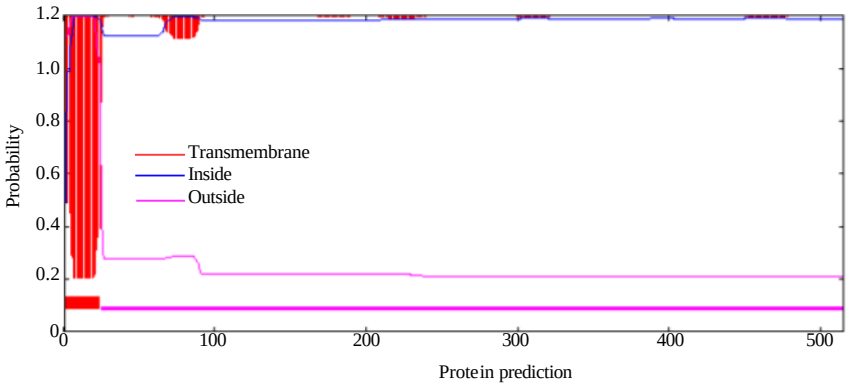

Fig. 4: The transmembrane protein prediction of tobacco flavonoid 3'-hydroxylase protein

Based on the results of the alignment of different species of flavonoid 3'-hydroxylase proteins, a phylogenetic tree was constructed using the ClustalW Software (http://www.ebi.ac.uk/clustalw) as shown in Fig. 5. The phylogenetic analysis revealed that the tobacco flavonoid 3'-hydroxylase gene has a closer genetic relationship with that of *Lycopersicon esculentum*.

Tissue expression profile: Tissue expression profile analysis was carried out and results revealed that the tobacco flavonoid 3'-hydroxylase gene was highly expressed in leaf and flower but hardly expressed in root and stem (Fig. 6).

Comparative genomics research has revealed that virtually all (99%) of the protein-coding genes in humans

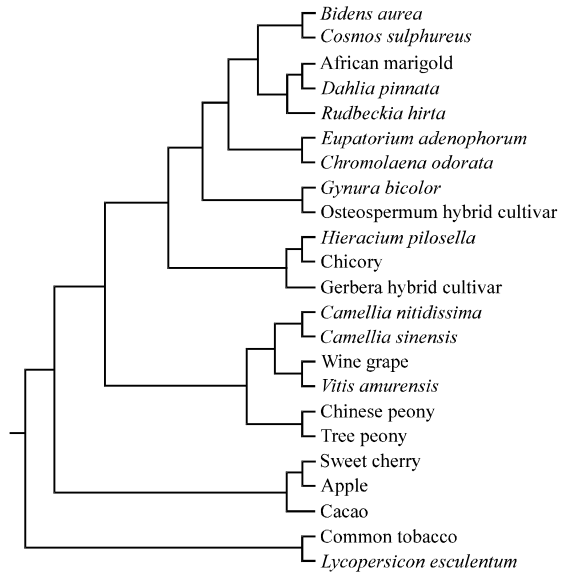

Fig. 5: The phylogenetic tree for five kinds of flavonoid 3'-hydroxylase genes

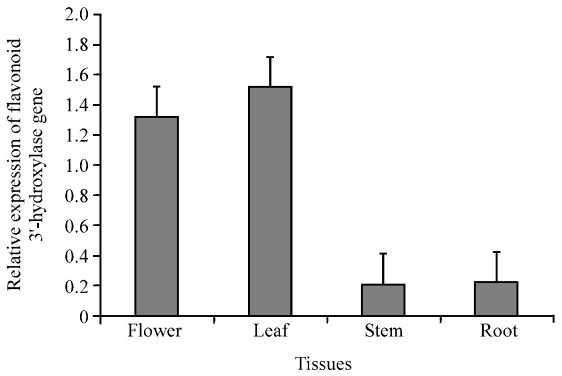

Fig. 6: Expression analysis of flavonoid 3'-hydroxylase gene mRNA in various tissues

align with homologs in mouse and over 80% are clear 1:1 orthologs for human and mouse both belong to mammalian (Hardison, 2003; Liu, 2009). This extensive conservation in protein-coding regions implied that this conservation of protein-coding sequences may be expected in tobacco and other plants of Solanaceae. From the sequence analysis of flavonoid 3'-hydroxylase genes, it can be seen that the coding sequences of flavonoid 3'-hydroxylase genes were highly conserved in two Solanaceae plants-tobacco and *Lycopersicon esculentum*.

The phylogenetic tree analysis revealed that the tobacco flavonoid 3'-hydroxylase gene has a closer genetic relationship with that of *Lycopersicon esculentum*. This implied that researchers can use *Lycopersicon esculentum* as model organism to study the tobacco flavonoid 3'-hydroxylase gene or use tobacco as model organism to study the *Lycopersicon esculentum* flavonoid 3'-hydroxylase gene.

From the tissue distribution analysis in the experiment it can be seen that flavonoid 3'-hydroxylase gene was highly expressed in leaf and flower. For flavonoid 3'-hydroxylase had been characterized to be required for flavonoid biosynthesis, the suitable explanation for this under current conditions is that flavonoid biosynthesis is mainly existed in leaf and flower.

CONCLUSION

Researchers first isolated the tobacco flavonoid 3'-hydroxylase gene and performed necessary sequence analysis and tissue expression profile analysis. These will establish the primary foundation of utilization of tobacco flavonoids in the future.

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