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# Molecular Characterization and Expression Pattern of a Novel Flavonol Synthase gene

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**Abstract:** Flavonols have significant health benefits for animals and humans. Flavonol synthase has been characterized to be required for flavonol biosynthesis. The complete coding sequence of tobacco Flavonol synthase gene was amplified by RT-PCR. The complete coding sequence of tobacco Flavonol synthase gene was 951 bp which encodes a protein of 316 amino acids. Sequence analysis revealed that the flavonol synthase of tobacco shares high homology with the flavonol synthase of *Lycopersicon esculentum* (69%), castor bean (64%), cucumber (58%) and chickpea (57%). Phylogenetic tree analysis revealed that the tobacco Flavonol synthase gene has a closer genetic relationship with that of *Lycopersicon esculentum*. Expression profile was studied and the results indicated that tobacco Flavonol synthase gene was highly expressed in in leaf and flower. These results established the primary foundation of utilization of tobacco flavonols in the future.

**Key words:** Tobacco, gene, flavonol synthase, expression pattern, encode

## INTRODUCTION

Flavonoids are a major class of plant secondary metabolites including chalcones, flavanones, dihyroflavonols and flavans, anthocyanins, flavones and 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).

Flavonols are the utmost important parts of flavonoids. Recent researches also showed that flavonol-rich dark cocoa significantly decreases plasma endothelin-1 and improves cognition in urban children (Calderon-Garciduenas *et al*., 2013) and dietary flavonol epicatechin can prevent the onset of type 1 diabetes in nonobese diabetic mice (Fu *et al*., 2013). Flavonol synthase has been characterized to be required for flavonoid biosynthesis. It catalyses dihydroflavonols into flavonols which presents a key branch of anthocyanins biosynthesis (Zhou *et al*., 2013).

Although, flavonol synthase play important roles in flavonoid biosynthesis until today, the tobacco Flavonol synthase 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 flavonols 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).

**Isolation of the coding sequence:** RT-PCR was performed to amplify complete coding sequences of tobacco Flavonol synthase gene using the cDNA obtained from the pooled tissues above. The 20 μL reaction system was: 2.0 μL cDNA, 2.0 μL 2 mM mixed dNTPs, 2.0 μL 10×Taq

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Table 1: PCR primers for tobacco Flavonol synthase gene isolation

| Gene | Primer sequence | Ta/°C | Length/bp |
|---|---|---|---|
| Flavonol synthase | Forward:5'-ATGGCTAGTTTTGATATTCCA-3' | 50 | 951 |
| | Reverse:5'-CTAATTAGAAATTGAATAGTGG-3' | | |

Table 2: qRT-PCR primers for tobacco Flavonol synthase, actin genes and annealing temperature

| Gene | Primer sequence | Ta/°C | Length/bp |
|---|---|---|---|
| Flavonol synthase | Forward:5'-CCTCTGGCTTCAATGTCT-3' | 56 | 250 |
| | Reverse:5'-CTGTGGTAATGGCTCAAC-3' | | |
| Actin | Forward:5'-CCATTCTTCGTTTGGACCTT -3' | 56 | 257 |
| | Reverse:5'-TTCTGGGCAACGGAACCT-3' | | |

DNA polymerase buffer, 1.2 μL 25 mM $MgCl_2$, 1.0 μL 10 mM forward primer, 1.0 μL 10 mM reverse primer, 2.0 units of Taq DNA polymerase (1 U/1 μL) and 9.8 μL sterile water. The primers for tobacco Flavonol synthase gene isolation were designed based on the tobacco EST sequences (GeneBank numbers BP531065 and AM788105) which are highly homologues with the coding sequence of Flavonol synthase gene of *Lycopersicon esculentum* (Table 1). The PCR program initially started with a 94°C denaturation for 4 min, followed by 35 cycles of 94°C/1 min, 56°C (Table 1)/1 min, 72°C /1 min then 72°C extension for 10 min, finally 4°C to terminate the reaction.

**Quantitative real time PCR (qRT-PCR) for tissue expression profile analysis:** The 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, 56°C for 15 sec (Table 2), 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

**Isolation result for tobacco Flavonol synthase gene:** For tobacco Flavonol synthase gene through RT-PCR with pooled tissue cDNAs, the resulting PCR products were 951 bp (Fig. 1).

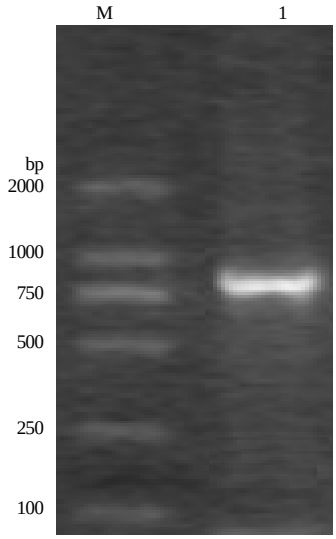


Fig. 1: PCR result for tobacco Flavonol synthase gene. M DL2000 DNA markers; 1 = PCR product for tobacco Flavonol synthase gene

**Sequence analysis:** The 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 tobacco gene and it was then deposited into the Genbank database (Accession No: KF856283).

The sequence prediction was carried out using the GenScan Software and results showed that the 951 bp cDNA sequence represents one single gene which encodes 316 amino acids. The theoretical isoelectric point (pI) and Molecular weight (Mw) of the deduced proteins of this tobacco gene were also computed using the Compute pI/Mw Tool (http://www.expasy.org/tools/pi_tool.html). The pI of tobacco flavonol synthase is 6.13. The molecular weight of this putative protein is 35842.63 (Fig. 2).

Further BLAST analysis of these proteins revealed that tobacco flavonol synthase has high homology with the flavonol synthase of *Lycopersicon esculentum* (Accession No: XP_004236012, 69%), castor bean

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ATGGCTAGTTTTGATATTCCAACGATTGATGTTTCTCCATTTTTGAAATCAGAGGACGATGAAGATGGGAAAAGGAAGGTTATCGAGCGA
 M  A  S  F  D  I  P  T  I  D  V  S  P  F  L  K  S  E  D  D  E  D  G  K  R  K  V  I  E  R
ATAAGGGAAGCCTGTGTTAACTATGGCTTCTTCCAGATTGAGAATCATGGAATTCCTTTGGGGTTGCTGAGCAAAACTATGGATTTGTAC
 I  R  E  A  C  V  N  Y  G  F  F  Q  I  E  N  H  G  I  P  L  G  L  L  S  K  T  M  D  L  Y
CAGACTTTCTTTGCTTGTTCAGATGAAGATAAATTATTGGTCTCTCCAAAACCCGGTTCATCAATACCAGCTGGTTATTTGAAAAGCCCA
 Q  T  F  F  A  C  S  D  E  D  K  L  L  V  S  P  K  P  G  S  S  I  P  A  G  Y  L  K  S  P
CAAAATTCAGCGGAGAAGAATGAGCACTTCGTCTTTCTTCCTCCATCCTCTGGCTTCAATGTCTATCCCAACAATCTACCTCATTTGAAA
 Q  N  S  A  E  K  N  E  H  F  V  F  L  P  P  S  S  G  F  N  V  Y  P  N  N  L  P  H  L  K
CAAGTTTTGGAGGAGATGATTTCGCGCTTCACCAAATTAGGAATGCTATTGGAGAGCATCATAAGTGAGTGTTTGGGCCTCCCTCCTAAC
 Q  V  L  E  E  M  I  S  R  F  T  K  L  G  M  L  L  E  S  I  I  S  E  C  L  G  L  P  P  N
ACCCTTGCAAAATTCAACGATGATCGGTCATGGGACTTCTTGATTGGTCTCCATTATTTTCCAGCAACAGAAGCTGAAAATAATGGAAAA
 T  L  A  K  F  N  D  D  R  S  W  D  F  L  I  G  L  H  Y  F  P  A  T  E  A  E  N  N  G  K
TCTGCACATGAAGATGGCAACTGCATTACTTTTGTCTACCAGGATGAAGTCGGAGGCCTTCAAGTGCACAAAGATGGGCAGTGGATCCCT
 S  A  H  E  D  G  N  C  I  T  F  V  Y  Q  D  E  V  G  G  L  Q  V  H  K  D  G  Q  W  I  P
ATAGCACCTTCCAAAGACAAACTAGTTGTTAACATTGGTGATATTATTCAGGTGTTAAGCAACAACAAATTCAAGAGTGCAACACATAGA
 I  A  P  S  K  D  K  L  V  V  N  I  G  D  I  I  Q  V  L  S  N  N  K  F  K  S  A  T  H  R
GTGGTGAGATCGAAGGGGAAAAGCCGTTACTCGTATGCATTCTTCTATAACTTGCACGGAGACAAGTGGGTTGAGCCATTACCACAGTTT
 V  V  R  S  K  G  K  S  R  Y  S  Y  A  F  F  Y  N  L  H  G  D  K  W  V  E  P  L  P  Q  F
ACAGAGGAAATTGGAGAATCGCCCAAGTACAGAGGATCCCTCTTCAAAGAATATGCGCAGCTAAGAGTCAAAAACAGGACTCATCCACCA
 T  E  E  I  G  E  S  P  K  Y  R  G  S  L  F  K  E  Y  A  Q  L  R  V  K  N  R  T  H  P  P
ACTAGACCTGAAGATCTCATCCATATAACCCACTATTCAATTTCTAATTAG
 T  R  P  E  D  L  I  H  I  T  H  Y  S  I  S  N  *
```

Fig. 2: The complete coding sequence of tobacco Flavonol synthase gene and its encoding amino acids. *indicates the stop codon

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Castor bean                 ----------MANWSIPTVDLSPFFK-EDDKDGKKKAMETISQAC
Chickpea                    MLRGYICEEILNQEMVEPSSIPTVDLSVFLR-EKE-DGKKKAMETITKAC
Cucumber                    ----------MAKDTGIPTVDLSVFSA-QNETEAKKKAFETIYQAC
Common tobacco              ----------MASFDIPTIDVSPFLKSEDDEDGKRKVIERIREAC
Lycopersicon esculentum     ----------MASFDIPTIDVSPFITSEENEEGKKKAIEQMREAC
                                      .***:*:* *   :.: :.*:*.:* : :**

Castor bean                 SEYGFFQIENHGVPLELMKQALKLSKEFFDFSYEEKRKYSPESGAPLPAG
Chickpea                    SEYGFFQIINHGVSLDLMKQAIELSKTFFDYSDEEKNKSSPLSNSPLPAG
Cucumber                    SSYGFFQIVNHGVPIEFLEEALELSRTFFHYPDDIKLKYSSKPGAPLLAG
Common tobacco              VNYGFFQIENHGIPLGLLSKTMDLYQTFFACSDEDKLLVSPKPGSSIPAG
Lycopersicon esculentum     VNYGFFQIANHGIPLDLLSRIMDMYKTFFACSDEEKLSVPSD-------N
                             .****** ***:.: ::.. :.: : **  . : *   ..       .

Castor bean                 YSKQPEHSPDKNEYVLVFPPGSGFNVYPPNPPGFREVLEGFFSYLTKTGS
Chickpea                    YGRQHMHSPDKNEYLLLFPPWSNFNVYPQNPPQFREAIEELFVQMSKIAV
Cucumber                    FNKQKKNCVDKNEYVLVFPPGSNYNIYPQEPPQFKELLEEMFKKLSKVCL
Common tobacco              YLKSPQNSAEKNEHFVFLPPSSGFNVYPNNLPHLKQVLEEMISRFTKLGM
Lycopersicon esculentum     YFKSTKKSAGTYEQLLFHLSSSGFNVCPENPPRFKQVLEEMASHFTNLGF
                            : :.  :.  . * .:.  . *.:*: * : * ::: :* :   :::

Castor bean                 LIESIINECLGLPHNFLKAFNHDRSWDFLVALRYFPATKSENNGLTDHED
Chickpea                    IMENIINDCLGLPSDFLKEFNQDRSWDLMSFKRYFPASKEENVGIAEHED
Cucumber                    LLESIVNESLGLPPDFLKQYNNDRSWDFMTTLYYFSATEEGENGLTHHED
Common tobacco              LLESIISECLGLPPNTLAKFNDDRSWDFLIGLHYFPATEAENNGKSAHED
Lycopersicon esculentum     VLGRIISECLGIPPNFLANYRNDQAKDFLLGIHYSPATEAENVGKSAHKD
                            ::  *:.:.**:* : *  :..*:: *::    * .*::  : * : *:*

Castor bean                 GNCLIFVFQDEAAGLQVRKNGEWIPVAPTENSIVVNVGDIIQVLSNNKFK
Chickpea                    GNCLIFVVQDGVGGLQVLRNGEWIPVVPAEGTIVVNVGDVIQVLSNKKFK
Cucumber                    GNCITLVFQDDTGGLQVRKDGEWIPVVPVEGAIVVNIGDVIQVLSNKKFK
Common tobacco              GNCITFVYQDEVGGLQVHKDGQWIPIAPSKDKLVVNIGDIIQVLSNNKFK
Lycopersicon esculentum     PGCITILYQPEVGGLQVQKDEQWIPIAPSKDKLVVNIGDVIQVLSNNKFK
                             .*:*:: *  ..**** :: :***:.* :. :***:**:******:***
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Fig. 3: The alignment of the proteins encoded by tobacco and other Flavonol synthase genes

(accession No: XP_002511567, 64%), cucumber (accession No: XP_004137405, 58%) and chickpea (accession No: XP_004499425,57%) (Fig. 3). Its conserved domains was identified as 2OG-Fe(II) oxygenase superfamily (2OG-FeII_Oxy) (Fig. 4). Based on the results of the alignment of different flavonol synthase proteins, a

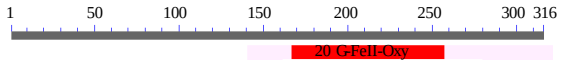


Fig. 4: The putative 2OG-Fe(II) oxygenase superfamily (2OG-FeII_Oxy) domain of the protein encoded by tobacco Flavonol synthase gene

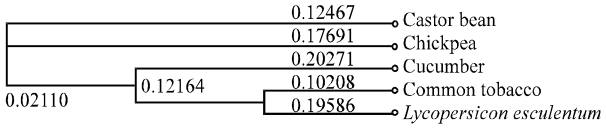


Fig. 5: The phylogenetic tree for five kinds of Flavonol synthase genes

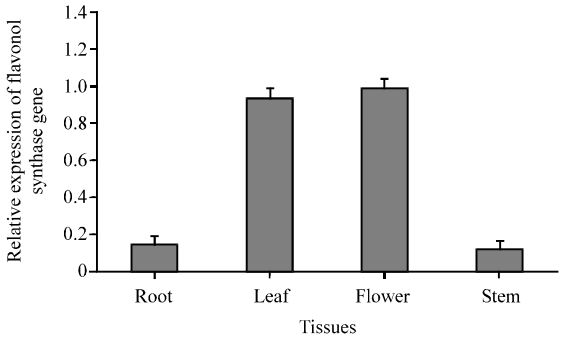


Fig. 6: Expression analysis of Flavonol synthase gene mRNA in various tissues

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 Flavonol synthase 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 Flavonol synthase 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 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 Flavonol synthase genes, it can be seen that the coding sequences of Flavonol synthase genes were highly conserved in two solanaceae plants-tobacco and *Lycopersicon esculentum* but bot conserved in other plants.

The phylogenetic tree analysis revealed that the tobacco Flavonol synthase 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 Flavonol synthase gene or use tobacco as model organism to study the *Lycopersicon esculentum* flavonol synthase gene.

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

## CONCLUSION

Researchers first isolated the tobacco Flavonol synthase gene and performed necessary sequence analysis and tissue expression profile analysis. These will establish the primary foundation of utilization of tobacco flavonol in the future.

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