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Initial Characterization and Expression Pattern Analysis of Tobacco (*Nicotiana Tabacum*) 2-Hydroxyisoflavanone Dehydratasedase Gene

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Abstract. The complete mRNA sequence of one tobacco (nicotiana tabacum) gene—2-hydroxyisoflavanone dehydratasedase, was amplified using the rapid amplification of cDNA ends methods based on one tobacco EST. The full-length tobacco 2-hydroxyisoflavanone dehydratasedase gene mRNA was 1,278bp containing a 966 bp open reading frame, which encodes a protein of 321 amino acids. Sequence analysis revealed that the 2-hydroxyisoflavanone dehydratasedase of tobacco shares high homology with the 2-hydroxyisoflavanone dehydratasedase of nicotiana tomentosiformis(99%), capsicum annuum(78%), potato(75%), lycopersicon pennellii(73%) and lycopersicon esculentum(72%). BLAST analysis within the tobacco high throughout genomic sequences database revealed that this gene has no intron and is a single exon gene. Results also showed that tobacco 2-hydroxyisoflavanone dehydratasedase gene has a closer genetic relationship with the 2-hydroxyisoflavanone dehydratasedase gene of nicotiana tomentosiformis. Tissue expression profile analysis revealed that the tobacco 2-hydroxyisoflavanone dehydratasedase gene was highly expressed in leaf and flower, but moderately expressed in root and stem. Our experiment established the foundation for further research on this tobacco gene.

1 Introduction

Isoflavonoids are commonly found in leguminous plants and have significant health benefits for animals and humans[1-3]. Isoflavonoids are the characteristic metabolites of the Leguminosae, the third largest family of the higher plants. Isoflavonoids play significant roles in the adaptation of the producer plants to their biological environments as, for example, defense substances (phytoalexins) and a host signal to symbiotic nitrogen-fixing rhizobial bacteria [4-5].

2-hydroxyisoflavanone dehydratasedase is a member of a large carboxylesterase family, of which plant proteins form a monophyletic group and some are assigned defensive functions with no intrinsic catalytic activities identified.

It has broader specificity to both 4′-hydroxylated and 4′-methoxylated 2-hydroxyisoflavanones and involves in leguminous isoflavone biosynthesis[4].

Although 2-hydroxyisoflavanone dehydratasedase play important roles in leguminous isoflavone biosynthesis, until today, the tobacco 2-hydroxyisoflavanone dehydratasedase gene has not been reported yet. In present experiment, we 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 understanding this tobacco gene.

2 Material and mehods

2.1 Samples collection, RNA extraction and first-strand cDNA synthesis

Tobacco plants (Chinese commercial 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℃[6]. Total RNA extraction and first-strand cDNA synthesis for these tissue samples were performed as the methods describe by Li et al.[7].

2.2 5’and 3’-RACE

5’- and 3’-RACE were performed as the instructions of BD SMARTTM RACE cDNA Amplification Kit (BD science, USA). For the tobacco 2-hydroxyisoflavanone dehydratasedase gene, the gene specific primers (GSPs) were designed based on the coding sequence information from potato 2-hydroxyisoflavanone dehydratasedase gene

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and its highly homologous tobacco EST sequence: EB429241.

5'-RACE GSP:

5'- CAGCACTATCCCCGGCTATGAATAA-3'

3'-RACE GSP:

5'- ACTTTATCAAGACTGCTGGGATGCC-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, 65°C: 30 sec and 72°C: 3 min, finally with 25 cycles of 94°C: 30 sec, 67°C: 30 sec and 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. At least five independent clones were sequenced for each PCR product.

2.3 Quantitative real time PCR (qRT-PCR) for tissue expression profile analysis

qRT-PCR for evaluating the level of mRNA for 2-hydroxyisoflavanone dehydratasedasegene was performed on the ABI Prism 7300 Sequence Detection Systems (Applied Biosystems, Foster City, CA, USA). PCR reactions for each sample were carried out in 25μl reaction volume containing 1μl 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 s, 52°C for 15 s (Table 1) and 72°C for 20 s. For each sample, reactions were set up in triplicate to ensure the reproducibility of the results. The gene relative expression levels were quantified relative to the expression of the reference gene, actin (GenBank Accession No. GQ339768) by employing the $2^{-\Delta\Delta Ct}$ value model[6,8].

2.4 Sequence analysis

The cDNA sequence prediction was conducted using GenScan software (http://genes.mit.edu/GENSCAN.html).

The protein prediction and analysis were performed using the Conserved Domain Architecture Retrieval Tool of BLAST at the National Center for Biotechnology Information (NCBI) server (http://www.ncbi.nlm.nih.gov/BLAST) and the Clustal Omega software (http://www.ebi.ac.uk/Tools/msa/clustalo/).

The theoretical isoelectric point (pI) and molecular weight (Mw) of the deduced protein of the tobacco gene was computed using the Compute pI/Mw Tool (http://www.expasy.org/tools/pi_tool.html).

3 Results

3.1 RACE results for tobacco 2-hydroxyisoflavanone dehydratasedase gene

For tobacco 2-hydroxyisoflavanone dehydratasedase gene, through 5'-RACE, one PCR product of 590bp was obtained. The 3'-RACE product was 813bp. These products were then cloned to T-vector and sequenced. Taken together, a 1,278-bp cDNA complete sequence was finally obtained (Figure 1).

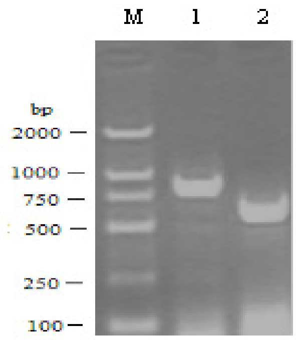

Figure 1. RACE results for tobacco 2-hydroxyisoflavanone dehydratasedase gene. M DL2000 DNA markers; 1,3'-RACE product for tobacco 2-hydroxyisoflavanone dehydratasedase gene; 2, 5'-RACE product for tobacco 2-hydroxyisoflavanone dehydratasedase gene

3.2 Sequence analysis

These 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 number: KJ438809).

The sequence prediction was carried out using the GenScan software and results showed that the 1,278-bp cDNA sequence represents one single gene which encodes 321amino acids (Figure 2). The pI of tobacco 2-hydroxyisoflavanone dehydratasedase is 5.32. The molecular weight of this putative protein is 35768.57. BLAST analysis within the tobacco high throughout genomic sequences database revealed that this gene has no intron and is a single exon gene.

Further BLAST analysis of this protein revealed that tobacco 2-hydroxyisoflavanone dehydratasedase has high homology with the 2-hydroxyisoflavanone dehydratasedase of nicotiana tomentosiformis (Accession number: XP_009624702, 99%), capsicum annuum (Accession number: XP_016573350, 78%), potato (Accession number: XP_006355549, 75%), lycopersicon pennellii (Accesession number: XP_015076742, 73%) and lycopersicon esculentum(Accesession number: NP_001316336, 72%) (Figure 3). Its conserved domain was identified as Abhydrolase superfamily.

The 3-D structural evidence of the putative conserved domain is also presented in figure 4. Based on the results of the alignment of different species of 2-hydroxyisoflavanone dehydratasedase proteins, a phylogenetic tree was constructed using the Clustal Omega software, as shown in Figure5 . The phylogenetic tree analysis revealed that the tobacco 2-hydroxyisoflavanone dehydratasedase gene has a closer genetic relationship with that of nicotiana tomentosiformis.

Table1. qRT-PCR primers for tobacco 2-hydroxyisoflavanone dehydratasedase, actin genes and annealing temperature

Gene	Primer sequence	Ta/ °C	Length/(bp)
2-hydroxyisoflavanone dehydratasedase	Forward : 5'- AAGTCTGGCGGAGATGTG-3' Reverse: 5'-AAAACGACGATGAGGAGG-3'	52	182
Actin	Forward :5'-CCATTCTTCGTTTGGACCTT -3' Reverse: 5'- TTCTGGGCAACGGAACCT-3'	56	257

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ACACAAGATTTAACATTTCATTCCCATAATCTCTTTGTGCATACTCTGTTCTGTTCCTAGTATTTCTCTGTGTTTCTCCAATATGACGTTTTCC
                                                                                  M  T  F  S
GAAAACGACGATGAGGAGGTGATCACTGATTTCCACCCTTACTACCGACTCTATAAAAACGGCCGAGTCCATCGTTTTACGAACTTTACGGC
 E  N  D  D  E  E  V  I  T  D  F  H  P  Y  Y  R  L  Y  K  N  G  R  V  H  R  F  Y  E  L  Y  G
ATAGTTGAAGTTCCTCCCTCGTTAGAAGATCCAGACACCGGCGTTTCATCCAAAGACGTGACCATTTCCCCTCACATCTCCGCCAGACTTTAC
 I  V  E  V  P  P  S  L  E  D  P  D  T  G  V  S  S  K  D  V  T  I  S  P  H  I  S  A  R  L  Y
CTTCCCAAAAACACTATCACCGATCAAAAGCTACCCGTCTTAGTTTACTACCACGGCGGAGGACTAGTTGCCGGATCCGCCTTCTTCCAGACG
 L  P  K  N  T  I  T  D  Q  K  L  P  V  L  V  Y  Y  H  G  G  G  L  V  A  G  S  A  F  F  Q  T
GAACACCGTTACCTCAACCATTTGGTTTCTGAATCAAAATGCATTGCTATTTCTGTGAATTATCGCCTTGCCCCAGAACATGACCTGCCCACA
 E  H  R  Y  L  N  H  L  V  S  E  S  K  C  I  A  I  S  V  N  Y  R  L  A  P  E  H  D  L  P  T
CTTTATCAAGACTGCTGGGATGCCCTTCAGTGGGTGGCTTCACACGCCGCCGGTAAAGACGCCGAACCGTGGATAGCAAACCATGGTGATTTT
 L  Y  Q  D  C  W  D  A  L  Q  W  V  A  S  H  A  A  G  K  D  A  E  P  W  I  A  N  H  G  D  F
AACAGATTATTCATAGCCGGGGATAGTGCTGGGGGTAATATTGTCTTTAACATGACCATGAGAGCTGGTAGGGAGAGCTTAAATGGAGGCGTG
 N  R  L  F  I  A  G  D  S  A  G  G  N  I  V  F  N  M  T  M  R  A  G  R  E  S  L  N  G  G  V
AAACTCGTCGGTGCCATCTTTGCTTTCCCTTTCTTCTTGATCCCATCTGTCGAAAACATTGAGGGGTCTTTGATTTACAAGCTTTGGAATACT
 K  L  V  G  A  I  F  A  F  P  F  F  L  I  P  S  V  E  N  I  E  G  S  L  I  Y  K  L  W  N  T
ATTTGTCCACCATCTGAACAGGGAATTAATAGCCCAATGGTTAATCCAGTTTCAGAAATATGCCCAAGCTTGTCTGAGTTAGGTTGCTCAAGG
 I  C  P  P  S  E  Q  G  I  N  S  P  M  V  N  P  V  S  E  I  C  P  S  L  S  E  L  G  C  S  R
CTTTTCGTGTGTGCAGGAAAGAAAGATGAGCTTGTCCCAAGTGAAATTGTGAGTCGATTCGCTGAAGCTGTGAAGAACAGTGGATGGAAAGGT
 L  F  V  C  A  G  K  K  D  E  L  V  P  S  E  I  V  S  R  F  A  E  A  V  K  N  S  G  W  K  G
GAATTAGAGTTCATTGAGGTTGAAGGTGAAGGTCATTGCTTTCAGGCTGCTAATCCTGAAGCTGAGAAATCTAAAGATCTGATCAAGCGCATG
 E  L  E  F  I  E  V  E  G  E  G  H  C  F  Q  A  A  N  P  E  A  E  K  S  K  D  L  I  K  R  M
GCTTCTTTCATCCAACGCAAGTGATTACAATTCTGCAATTAGAGCTCTTAGTAATTATTATTAGATCTTCTAATCTTAAATTAGGCGATGAGTAT
 A  S  F  I  Q  R  K  *
ATATTTGAAGCATATTTTTGAGTGGATATGTTATTAGAACTGATTTACTGTATCCTAGGAGCGTTAAAATTTATTATAGTTTACGCTAAACTAAT
AGATGTATGATATGTTCTATACATGAATTTCAAGCATTTAATCATTGTTAAGTAAAAAAAAAAA
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Figure 2. The complete mRNA of tobacco 2-hydroxyisoflavanone dehydratasedase gene and its encoding amino acids *indicates the stop codon

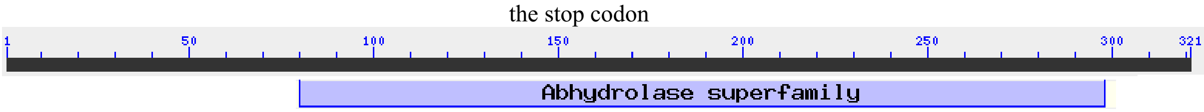

Figure 3. The putative Abhydrolase superfamily domain of the protein encoded by tobacco 2-hydroxyisoflavanone dehydratasedase gene

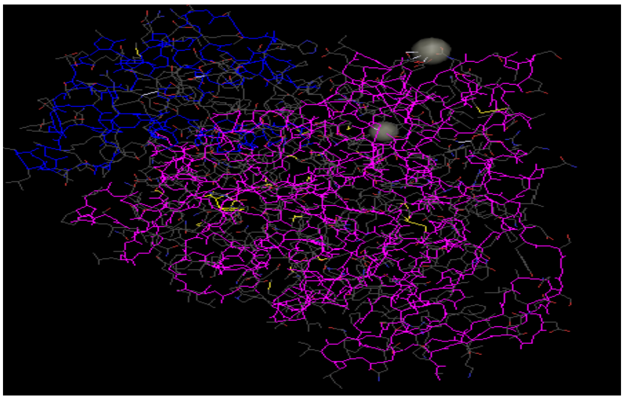

Figure 4. The 3-D structural evidence of the putative conserved domain of tobacco 2-hydroxyisoflavanone dehydratasedase protein

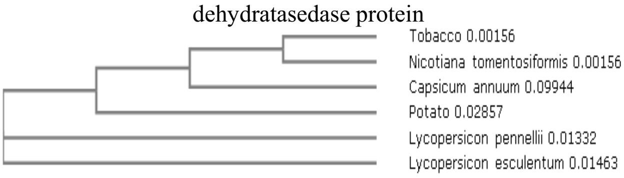

Figure 5. The phylogenetic tree for six kinds of 2-hydroxyisoflavanone dehydratasedase genes

3.3 Tissue expression profile

Tissue expression profile analysis was carried out and results revealed that the tobacco 2-hydroxyisoflavanone dehydratasedase gene was highly expressed in leaf and flower, but moderately expressed in root and stem (Figure 6).

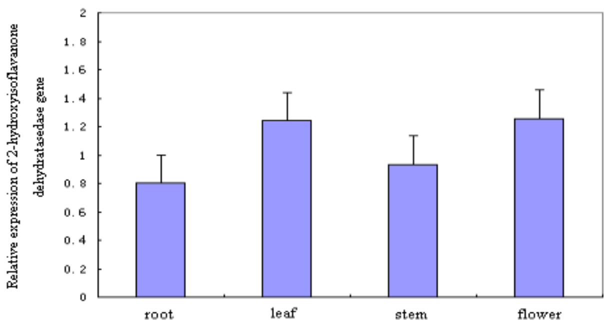

Figure 6. Expression analysis of 2-hydroxyisoflavanone dehydratasedase gene mRNA in various tissues

4 Conclusions

Comparative genomics research has revealed that the extensive conservation in protein-coding regions implied that this conservation of protein-coding sequences may be expected in tobacco and other plants[6,9]. From the sequence analysis of 2-hydroxyisoflavanone dehydratasedase genes, it can be seen that the coding sequences of 2-hydroxyisoflavanone dehydratasedase genes were highly conserved in some plants. This implied that we can use the some plants as model organisms and isolate some tobacco genes based on the coding sequence information of these plants. Isolation of the tobacco 2-hydroxyisoflavanone dehydratasedase gene in this experiment further validated that is an effective method.

The phylogenetic tree analysis revealed that the tobacco 2-hydroxyisoflavanone dehydratasedase gene has a closer genetic relationship with that of nicotiana tomentosiformis. This implied that we can use nicotiana tomentosiformis as model organism to study the tobacco 2-hydroxyisoflavanone dehydratasedase gene or use tobacco as model organism to study the nicotiana tomentosiformis 2-hydroxyisoflavanone dehydratasedase gene.

From the tissue distribution analysis in our experiment it can be seen that 2-hydroxyisoflavanone dehydratasedase gene was obviously differentially expressed in some tissues. The tobacco 2-hydroxyisoflavanone dehydratasedase gene was highly expressed in leaf and flower, but moderately expressed in root and stem. For 2-hydroxyisoflavanone dehydratasedase functions in leguminous isoflavone biosynthesis[4]. The suitable explanation for differential expression under current conditions is that the 2-hydroxyisoflavanone dehydratasedase related leguminous isoflavone biosynthesis was high in leaf and flower, but moderate in root and stem.

In conclusion, we first isolated the tobacco 2-hydroxyisoflavanone dehydratasedase gene and performed necessary sequence analysis and tissue expression profile analysis. This established the primary foundation for further research on this tobacco gene.

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