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Molecular Characterization and Expression Pattern of a Novel *NMNAT1* Gene

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Abstract: Nicotinamide adenine dinucleotide is important to the health of animals and humans. Nicotinamide Mononucleotide Adenylyltransferase 1 (*NMNAT1*) gene has been characterized to be required for nicotinamide adenine dinucleotide biosynthesis. In present experiment, the complete mRNA sequence of tobacco *NMNAT1* gene was amplified using the rapid amplification of cDNA ends methods. The full-length tobacco *NMNAT1* gene mRNA was 1,445 bp containing a 753 bp open reading frame which encodes a protein of 250 amino acids. BLAST analysis revealed that tobacco NMNAT1 protein shares high homology with the NMNAT1 of *Lycopersicon esculentum* (87%), wine grape (65%), soybean (65%), cacao (65%) and barrel medic (62%). Results also showed that tobacco *NMNAT1* gene has a closer genetic relationship with the *NMNAT1* gene of *lycopersicon esculentum*. The expression profile was studied and the results indicated that tobacco *NMNAT1* gene was highly expressed in leaf. These results established the primary foundation of utilizing tobacco nicotinamide adenine dinucleotide or NMNAT1 as drugs for animals and humans in the future.

Key words: Tobacco, gene, NMNAT1, expression pattern, dinucleotide

INTRODUCTION

Nicotinamide adenine dinucleotide is a dinucleotide which consists of two nucleotides: one nucleotide contains an adenine base and the other nicotinamide. Nicotinamide adenine dinucleotide is important to the health of animals and humans. Evidence indicates that the depletion of nicotinamide adenine dinucleotide results in axonal degeneration (Ding *et al*., 2013; Kaneko *et al*., 2006; Sasaki *et al*., 2006). Axonal degeneration is a common pathological feature of a variety of neuropathological disorders such as Alzheimer's disease, amyotrophic lateral sclerosis, Parkinson's disease and diabetic neuropathies (Ding *et al*., 2013; Fischer *et al*., 2004; Raff *et al*., 2002; Stokin *et al*., 2005). Moreover, nicotinamide adenine dinucleotide supplementation suppresses the development of axonal degeneration in traumatic injury, ischemia damage, autoimmune encephalomyelitis, p53-induced neuron apoptosis and radiation-induced immunosuppression (Ding *et al*., 2013; Luo *et al*., 2001; Klaidman *et al*., 2003; Sadanaga-Akiyoshi *et al*., 2003). The reduction of axonal degeneration by nicotinamide adenine dinucleotide is presumably due to its propensity to reduce oxidative stress or oxidative damage in the neurons (Ding *et al*., 2013; Zhang and Lindup, 1996; Kawai *et al*., 2006; Hipkiss, 2010). Addition of exogenous nicotinamide adenine dinucleotide can prevent mefloquine-induced neuroaxonal and hair cell degeneration through reduction of caspase-3-mediated apoptosis in cochlear organotypic cultures (Ding *et al*., 2013). As mentioned above, it can be seen that nicotinamide adenine dinucleotide is an important drug which has significant health benefits for animals and humans. Nicotinamide Mononucleotide Adenylyltransferase 1 (NMNAT1) is a central enzyme in nicotinamide adenine dinucleotide biosynthesis, catalyzing the condensation of nicotinamide mononucleotide or nicotinic acid mononucleotide with the AMP moiety of ATP to form nicotinamide adenine dinucleotide or NaAD (Muller *et al*., 2012). NMNAT1 can affect the rate of Wallerian degeneration in mice and drosophila (Zhao *et al*., 2011).

NMNAT1 gene has been identified from many plants such as soybean, tomato and wine grape. Until today, the tobacco *NMNAT1* gene has not been reported yet. In present experiment, researhers 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 utilizing tobacco nicotinamide adenine dinucleotide or NMNAT1 as drugs for animals and humans 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

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Table 1: qRT-PCR primers for tobacco *NMNAT1*, *Actin* genes and annealing temperature

Genes	Primer sequence	Ta (°C)	Length (bp)
NMNAT1	Forward: 5'-GTGGCGTTGAAAGTGGTT -3'	56	445
	Reverse: 5'-AGCGCAAGTGCATGTAAG -3'		
Actin	Forward: 5'-CCATTCTTCGTTTGGACCTT -3'	56	257
	Reverse: 5'-TTCTGGGCAACGGAACCT-3'		

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 *NMNAT1* gene, the Gene Specific Primers (GSPs) were designed based on one tobacco EST sequence: FG165797. 5'-RACE GSP: 5'- ACCGGCAATGA TCTTTTCAACATCC-3' 3'-RACE GSP: 5'- CATGGTCA TGCTTGTGTGTGGTCTG-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, 68°C 30 sec and 72°C 3 min, finally with 25 cycles of 94°C 30 sec, 68°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 *NMNAT1* gene was performed by the ABI Prism 7300 Sequence Detection Systems (Applied Biosystems, Foster City, CA, USA). About 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 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 $2^{-\Delta\Delta Ct}$ 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 theoretical Isoelectric point (pI) and Molecular weight (Mw) of the deduced protein was computed using the Compute pI/Mw Tool (http://www.expasy.org/tools/pi_tool.html). The protein analysis were carried out 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).

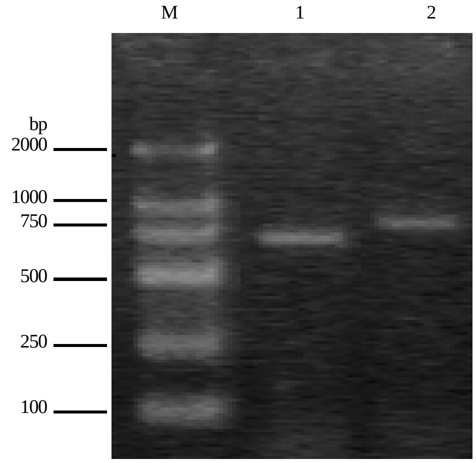

Fig. 1: RACE results for tobacco *NMNAT1* gene. M: DL2000 DNA markers; 1: 3'-RACE product for tobacco *NMNAT1* gene; 2: 5'-RACE product for tobacco *NMNAT1* gene

RESULTS AND DISCUSSION

RACE results for tobacco *NMNAT1* gene: For tobacco *NMNAT1* gene, through 5'-RACE, one PCR product of 864 bp was obtained. The 3'-RACE product was 731 bp. These products were then cloned to T-vector and sequenced. Taken together, a 1,445 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: KF856280).

The sequence prediction was carried out using the GenScan Software and results showed that the 1,445 bp cDNA sequence represents one single gene which encodes 250 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. The pI of tobacco NMNAT1, chloroplastic-like is 6.59. The molecular weight of this putative protein is 27899.90.

Further BLAST analysis of this protein revealed that tobacco NMNAT1 shares high homology with the NMNAT1 of *Lycopersicon esculentum* (Accession

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GAGACAGTGGCGTTGAAAGTGGTTATTTGTGCACTTCGCCCCACAAAAGGGAGTAAGTGCAAAGCCATAGTAGACTTGTTGAAAAGAGGGAT
CATCAAGCTAATGCGCGATTCCCCCAAATCCATTAACTATCTTTTATAGAATTTTTCACCTGCTGGGGTAACTCCTGCGCTCAAAGACCTTG
TCAAAGGCCATTTTCCGGTCAGGACTTTGCAAAGAAGCCAGCAACACAACTCTTGAATGCAATATTTGTTCCTTATCAGCTCTGCACAACAA
TAGGGTGTCATCCGCATACAAAGTATGACTGATATTGCTTTACCATGGGATAAGCTGTCTTTAGATTTAATAAAACAAGAGGAGGGGCAA
                         M  T  D  I  A  L  P  W  D  K  L  S  L  D  L  I  K  Q  E  E  G  Q
TCAAGTCCGGAAAGGAAGAAAAGGACATATGTAGTTCTTGTATCCACAGGAAGTTTCAATCCTCCTACTTACATGCACTTGCGCTGTTTT
 S  S  P  E  R  K  K  R  T  Y  V  V  L  V  S  T  G  S  F  N  P  P  T  Y  M  H  L  R  C  F
GAGTTGGCAAGAGATGCATTGACTTCAGAAGGGTTCTGCGTAATTGGAGGTTATATGTCACCAGTAAATGATGCATATAAGAAGAAGGGT
 E  L  A  R  D  A  L  T  S  E  G  F  C  V  I  G  G  Y  M  S  P  V  N  D  A  Y  K  K  K  G
CTTATATCTGCCGAGCATCGTGTTGCAATGTGCCAGTTAGCTTGTAAAAGCTCAGAATTCGTTATGACAGATCCCTGGGAGGCAAGCCAG
 L  I  S  A  E  H  R  V  A  M  C  Q  L  A  C  K  S  S  E  F  V  M  T  D  P  W  E  A  S  Q
GATAGCTATCAACGAACATTGACAGTTCTCTCCAGAATAAAGTCCGCTATCTGTGGTGGAAGTTGTCATCCAGTGACTCATGGTCATGCT
 D  S  Y  Q  R  T  L  T  V  L  S  R  I  K  S  A  I  C  G  G  S  C  H  P  V  T  H  G  H  A
TGTGTGTGGTCTGATCTGCTAGAATCTTTCAGCACGCCTGGAGTTTGGATACCTGAGCAGGTCAGGACCATATGTAGAGACTTTGGCTTG
 C  V  W  S  D  L  L  E  S  F  S  T  P  G  V  W  I  P  E  Q  V  R  T  I  C  R  D  F  G  L
GTTTGTGTCCGGAGAGGTGGTCAGGATGTTGAAAAGATCATTGCCGGTGATGATATTTTGAATGAATATAAGAAAAATATCAAAGTTGTG
 V  C  V  R  R  G  G  Q  D  V  E  K  I  I  A  G  D  D  I  L  N  E  Y  K  K  N  I  K  V  V
GATGAAGTAGTGCCTAATGGAATCAGTTCAACGGGATTAAGGGACTGCATCTCGAAAGGGTTCTCAGTGAAGTACTTGACAGCCGATGAA
 D  E  V  V  P  N  G  I  S  S  T  G  L  R  D  C  I  S  K  G  F  S  V  K  Y  L  T  A  D  E
GTAATTGATTATATGAAACAACATAACCTTTATAGAGGGCAATGTTCTAACAACTGAATCAGAAGCTCCTTTTAGGAGCTTGTTTTCTTAAA
 V  I  D  Y  M  K  Q  H  N  L  Y  R  G  Q  C  S  N  N  *
TAATTGTTGATACCTGTCCAAGAGTCGCTCATTTCTTTGAGCTGTGATTTTAGCAGCGAGGCTACTGTCCCTTCCTGCAAGAGCAGAAAGAT
GTATTCTTTGTACTTCCTTCAAGGACAAGAGATGAGGACGAACACTTGCTCATTCCAGTTGTGTCCTCTATCTATGTTCATTTGTGATGAAA
TCTATTTCTTTTTTGAAACAAGGGCTAGTTACAGATGCTCCAGCCAGTTGAGGGAGTAGATCTTGGCTTCATGTGATTATAACTTATAACAC
AAAAACCTCAAAATGTCTTTAAAAGTGCTATAATGTATTACTATTGTTTTTCTTGGATTACTTATTAAAAAAAAAAAAAAA
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Fig. 2: The complete mRNA of tobacco *NMNAT1* gene and its encoding amino acids. *indicates the stop codon

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Cacao                          -----MEIPLPLNKLSLPSIT---------NGDSVYVVLVSTGSFNPPTL
Wine grape                     MTSDTMDIPLPLEKLSLESIDE-DRSLETTNREKMYVALVATGSFNPPTN
Soybean                        -----MDVPLPRDKLALDLINN-EPSPANTSNSKIYVILVATGSFNPPTF
Barrel medic                   ---MVMDVPLPLDKLALQLINN-EPSPGNTSDGKIYIILVATGSFNPPTF
Common tobacco                 ----MTDIALPWDKLSLDLIKQEEGQSSPERKKRTYVVLVSTGSFNPPTY
Lycopersicon esculentum        -MSSKTDIALPLDKLSLDLIKQMEGQLSPE--KRTYAVLVSTGSFNPPTY
                                     ::.** :**:*  *               *  **:********

Cacao                          MHLRMFELARDALNSDGFCVIGGYMSPVNDAYKKKGLIAAEHRTELCNLA
Wine grape                     MHLRMFELARDALRSEGYCVIGGYMSPVNDAYKKRGLISAEHRIQMCDLA
Soybean                        MHLRMFELARDALNSDGYCVIGGYLSPVNDAYKKKGLISAEHRIQLCHLA
Barrel medic                   MHLRMFELARDALNSKGYCVIGGYMSPVNDAYKKKNLISADHRIQLCHLA
Common tobacco                 MHLRCFELARDALTSEGFCVIGGYMSPVNDAYKKKGLISAEHRVAMCQLA
Lycopersicon esculentum        MHLRCFELARDALTSEGICVIGGYMSPVNDAYKKKNLISAEHRVAMCQLA
                               **** ******** *.* ******:*********:.**:*:**  :*.**

Cacao                          CKSSEFIMVDPWEANQSTFQRTLTVLSRVKSFLTEGGLIPKESLKVMLVC
Wine grape                     CKSSEFIMVDPWEANQSTFQRTLTVLSRIKCSLCENGLIPRESLKVMLVC
Soybean                        CKSSDFIMVDPWEASQSTYQRTLTVLSRVHNSVCETGLVSQESLKVMLLC
Barrel medic                   CKSSEFVMVDPWEANQNTYQRTLTVLSRVHASICETGLISRESLKVMLVC
Common tobacco                 CKSSEFVMTDPWEASQDSYQRTLTVLSRIKSAICGGSCHPVTHGHACVWS
Lycopersicon esculentum        CKSSEFVMTDPWEASQDSYQRTLTVLSRIKSAISGGSLTSTNDLMVTLVC
                               ****:*:*.*****.*.::*********::  :   .  .     . : .

Cacao                          GSDLLQSFSIPGFWIPEQVRSICKDYGVVCIRREGQDVEKIITDDEILNE
Wine grape                     GSDLLESFGIPGFWITEQVMAICRDYGVVCIRREGQDVEKIISDNNILNE
Soybean                        GSDLLHSFSIPGFWIPDQVKTICKDYGVVCIPREGQDVEKTIFKDDILNE
Barrel medic                   GSDLLHSFGIPGFWIPDQVKSICRDYGVVCIRREGQNIEKTISDDNILNE
Common tobacco                 --DLLESFSTPGVWIPEQVRTICRDFGLVCVRRGGQDVEKIIAGDDILNE
Lycopersicon esculentum        GSDLLESFSTPGVWIPEQVRAICRDFGLVCIRRSGQDVEKIITGDEILNE
                                 ***.**. **.**.:** :**:*:*:**: * **::** *  ::****

Cacao                          NRDNIKIVDELVPNLISSTKVRECISRGLSIKYLTVDEVIDYIRKHHLYL
Wine grape                     NKGNIIVVDDLVPNQISSTRVRECISRQLSVKYLMEDRVIDYIKRHHLYS
Soybean                        NKDNIKVVNELVPNQISSTRVRDCIARGLSIKYLTADEVIDYIREQQLYL
Barrel medic                   NQANIEVVDELVPNQISSTRIRECIARGLSIKYLTADEVIDYTREHKLYL
Common tobacco                 YKKNIKVVDEVVPNGISSTGLRDCISKGFSVKYLTADEVIDYMKQHNLYR
Lycopersicon esculentum        YKKNIRVVDEVVPNGISSTGLRDCISKGLSVKYLTADEVIDYIKQHNLYK
                                : ** :*:::*** **** :*:**:: :*:***  *.**** :.::**

Cacao                          NLDEK-
Wine grape                     NSSER-
Soybean                        N-----
Barrel medic                   KSDDK-
Common tobacco                 GQCSNN
Lycopersicon esculentum        GQ----
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Fig. 3: The alignment of the proteins encoded by tobacco and other *NMNAT1* genes

number: XP_004244056, 87%), wine grape (Accession number: XP_002268571, 65%), soybean (Accession number: P_003544646, 65%), cacao (Accession number: EOY12017, 65%) and barrel medic (Accession number: XP_003617833, 62%) (Fig. 3). Based on the results of the alignment of different species of NMNAT1 proteins, a

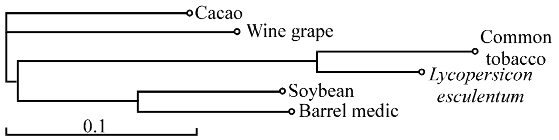

Fig. 4: The phylogenetic tree for six kinds of *NMNAT1* genes

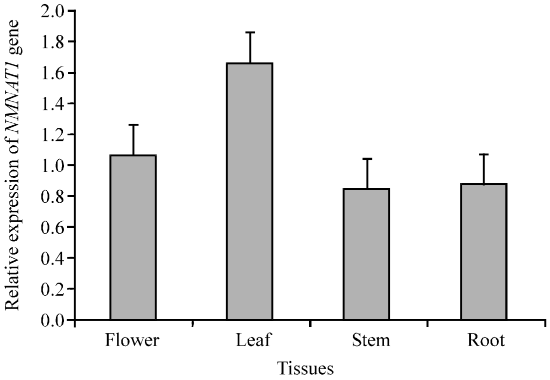

Fig. 5: Expression analysis of *NMNAT1* gene mRNA in various tissues

phylogenetic tree was constructed using the ClustalW Software as shown in Fig. 4. The phylogenetic analysis revealed that the tobacco *NMNAT1* 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 *NMNAT1* gene was highly expressed in leaf but moderately expressed in flower, root and stem (Fig. 5).

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 *NMNAT1* genes, it can be seen that the coding sequences of *NMNAT1* genes were highly conserved in two Solanaceae plants-tobacco and *Lycopersicon esculentum*.

The phylogenetic tree analysis revealed that the tobacco *NMNAT1* gene has a closer genetic relationship with that of *Lycopersicon esculentum*. This implied that resrarchers can use *Lycopersicon esculentum* as model organism to study the tobacco *NMNAT1*gene or use tobacco as model organism to study the *Lycopersicon esculentum NMNAT1*gene.

From the tissue distribution analysis in the experiment, it can be seen that *NMNAT1* gene was highly expressed in leaf. For NMNAT1 is a central enzyme in nicotinamide adenine dinucleotide biosynthesis (Muller *et al*., 2012), the suitable explanation for this under current conditions is that nicotinamide adenine dinucleotide biosynthesis is mainly existed in leaf.

CONCLUSION

Researchers first isolated the tobacco *NMNAT1* gene and performed necessary sequence analysis and tissue expression profile analysis. These will establish the primary foundation of using tobacco nicotinamide adenine dinucleotide or NMNAT1 as drugs for animals and humans in the future.

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