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Molecular Characterization and Expression Pattern of a Novel Nicotinamide Adenine Dinucleotide Transporter Gene

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Abstract: Nicotinamide adenine dinucleotide is a drug which is important to the health of animals and humans. Nicotinamide adenine dinucleotide transporter 1 gene have been characterized to be required for nicotinamide adenine dinucleotide bioactivety. In present experiment, the complete mRNA sequence of tobacco nicotinamide adenine dinucleotide transporter 1 gene was amplified using the rapid amplification of cDNA Ends Methods. The full-length tobacco nicotinamide adenine dinucleotide transporter 1 gene *mRNA* was 1,588 bp containing an 948 bp open reading frame which encodes a protein of 315 amino acids. BLAST analysis revealed that tobacco nicotinamide adenine dinucleotide transporter 1 protein shares high homology with the nicotinamide adenine dinucleotide transporter 1 of *Lycopersicon esculentum* (93%), wine grape (86%), soybean (81%), chickpea (79%) and foxtail millet (78%). Results also showed that tobacco nicotinamide adenine dinucleotide transporter 1 gene has a closer genetic relationship with the nicotinamide adenine dinucleotide transporter 1 gene of *Lycopersicon esculentum*. Prediction of transmembrane helices showed that tobacco nicotinamide adenine dinucleotide transporter 1 might be a transmembrane protein. The expression profile was studied and the results indicated that tobacco nicotinamide adenine dinucleotide transporter 1 gene was highly expressed in leaf. These results established the primary foundation of using tobacco nicotinamide adenine dinucleotide as drug for animals and humans in the future.

Key words: Tobacco, nicotinamide adenine dinucleotide transporter 1, expression pattern, protein, wine

INTRODUCTION

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). Evidence indicates that the depletion of the coenzyme nicotinamide adenine dinucleotide results in axonal degeneration (Ding *et al*., 2013; Kaneko *et al*., 2006; Sasaki *et al*., 2006). 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 *et al*., 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 adenine dinucleotide is synthesized outside the mitochondria and must be imported across the permeability barrier of the inner mitochondrial membrane (Todisco *et al*., 2006). Nicotinamide adenine dinucleotide transporter 1 has been characterized to be responsible for this transport activity (Todisco *et al*., 2006). Nicotinamide adenine dinucleotide transporter 1 gene has been identified from many plants such as soybean, tomato and wine grape. Until today, the tobacco nicotinamide adenine dinucleotide transporter 1 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 using tobacco nicotinamide adenine dinucleotide as drug for animals and humans in the future.

MATERIALS AND METHODS

Samples collection, RNA extraction and first-strand cDNA synthesis: Tobacco plants (Chinese local variety

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Table 1: The qRT-PCR primers for tobacco nicotinamide adenine dinucleotide transporter 1, *actin* genes and annealing temperature

Genes	Primer sequence	Ta (°C)	Length (bp)
Nicotinamide adenine dinucleotide transporter 1	Forward: 5'-ATGCGACTGTGGTTCTGT-3'	51	211
	Reverse: 5'-TGTAGCCGCTATTACTCC-3'		
Actin	Forward: 5'-CCATTCTTCGTTTGGACCTT-3'	56	257
	Reverse: 5'-TTCTGGGCAACGGAACCT-3'		

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: 5' and 3'-RACE were performed as the instructions of SMART™ RACE cDNA Amplification kit. For the tobacco nicotinamide adenine dinucleotide transporter 1 gene, the Gene Specific Primers (GSPs) were designed based on one tobacco EST sequence: CK294350. 5'-RACE GSP: 5'-CATGACTAACACCAGCCAAAGCAGG-3'3'-RACEGSP: 5'-GCCTGCTTTGGCTGGTGTTAGTCAT-3'.

RACE touch down 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 nicotinamide adenine dinucleotide transporter 1 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 $2^{-\Delta\Delta Ct}$ 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 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). The prediction of transmembrane helices in protein was carried out by the TMHMM Server V. 2.0 (http://www.cbs.dtu.dk/services/TMHMM/).

RESULTS AND DISCUSSION

RACE results for tobacco nicotinamide adenine dinucleotide transporter 1 gene: For tobacco nicotinamide adenine dinucleotide transporter 1 gene, through 5'-RACE, one PCR product of 892 bp was obtained. The 3'-RACE product was 721 bp. These products were then cloned to T-vector and sequenced. Taken together, a 1, 588 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: KF856282).

The sequence prediction was carried out using the GenScan Software and results showed that the 1,588 bp cDNA sequence represents one single gene which encodes 315 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 nicotinamide adenine dinucleotide transporter 1, chloroplastic-like is 9.53. The molecular weight of this putative protein is 33871.19.

Further, BLAST analysis of this protein revealed that tobacco nicotinamide adenine dinucleotide transporter 1 shares high homology with the nicotinamide adenine dinucleotide transporter 1 of *Lycopersicon esculentum* (Accession number: XP_004247391, 93%), wine grape (Accession number: XP_002273574, 86%), soybean (Accession number: XP_003554533, 81%), chickpea (Accession number: XP_004495929, 79%) and foxtail millet (Accession number: XP_004962357, 78%) (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 nicotinamide adenine dinucleotide transporter 1 protein might be a transmembrane protein (Fig. 4). Based on the results of the alignment of different species of nicotinamide adenine dinucleotide transporter 1 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 nicotinamide adenine dinucleotide transporter 1 gene has a closer genetic relationship with that of *Lycopersicon esculentum*.

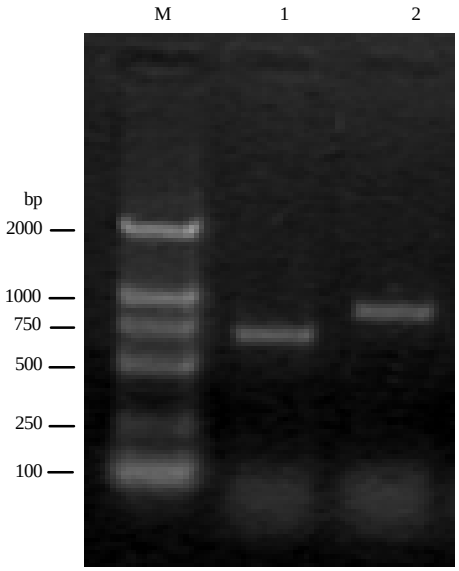

Fig. 1: RACE results for tobacco nicotinamide adenine dinucleotide transporter 1 gene. M: DL2000 DNA markers; 1: 3'-RACE product for tobacco nicotinamide adenine dinucleotide transporter 1 gene; 2: 5'-RACE product for tobacco nicotinamide adenine dinucleotide transporter 1 gene

Tissue expression profile: Tissue expression profile analysis was carried out and results revealed that the tobacco nicotinamide adenine dinucleotide transporter 1 gene was highly expressed in leaf but hardly expressed in flower, 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 nicotinamide adenine dinucleotide transporter 1 genes, it can be seen that the coding sequences of nicotinamide adenine dinucleotide transporter 1 genes were highly conserved in two solanaceae plants-tobacco and *Lycopersicon esculentum*. The phylogenetic tree analysis revealed that the tobacco nicotinamide adenine dinucleotide transporter 1 gene has a closer genetic relationship with that of *Lycopersicon esculentum*. This implied that researchers can use lycopersicon esculentum as model organism to study

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CCGAGAGCTGCCAAATTGGACCACCTCTCAGGCGATTTTAGATTCTTGATCTCATCTTCACTCAACAACCAACGTAGCCAAAACAAATCCCT
TTTGTGAAAAATTAAATCTTGAATTGCCCCTTTAATAGAAAACTTGTTTGGGGGCAATTCAATTTGGCGAGTGACTGGTGAAATTTCGTTAT
TTTGGAGTTATACCTACTGGCCATGCGACTGTGGTTCTGTGCGATCTCCCTCTTAAAAGAGGACTTTAAGAATCAAGTTCAAGTCTTTTATA
TCTCTGCAGCTGCTGTTTCCTTTGTAATTAATTGCCCTAGATTTAGCAATAGCAATGGCCGCTGATACTCATGGCCCTACTCCCAAAGGC
                                                        M  A  A  D  T  H  G  P  T  P  K  G
CTTCTTTGCAATGCTGGTGCTGGTGCCGCTGCTGGAGTAATAGCGGCTACATTTGTGTGCCCTTTAGATGTTATCAAGACTAGGTTGCAG
 L  L  C  N  A  G  A  G  A  A  A  G  V  I  A  A  T  F  V  C  P  L  D  V  I  K  T  R  L  Q
GTTCATGGGTTGCCAAAGATAGGAACTGCTAACGTTAGAGGTAGTCTTATAGTTGGGAGTTTGGAGCAGATATTTCAAAGGGAGGGGTTG
 V  H  G  L  P  K  I  G  T  A  N  V  R  G  S  L  I  V  G  S  L  E  Q  I  F  Q  R  E  G  L
CGTGGAATGTACAGAGGGCTTTCCCCAACAGTGCTAGCATTACTGCCAAACTGGGCGGTCTATTTTACTATATATGAACAGCTGAAAAGC
 R  G  M  Y  R  G  L  S  P  T  V  L  A  L  L  P  N  W  A  V  Y  F  T  I  Y  E  Q  L  K  S
TTTCTTGGCTCTGATGATGGAAGTCATCAGCTTTCGATAGGTGCAAACATGTTAGCTGCTTCCGGTGCCGGAGCTGCAACCACAATTGCA
 F  L  G  S  D  D  G  S  H  Q  L  S  I  G  A  N  M  L  A  A  S  G  A  G  A  A  T  T  I  A
ACAAATCCTCTCTGGGTTGTGAAGACACGTCTTCAGACTCAAGGAATGAAAGCAGGTCTCGAGCCTTACAGGGGTACATTATCTGCTTTA
 T  N  P  L  W  V  V  K  T  R  L  Q  T  Q  G  M  K  A  G  L  E  P  Y  R  G  T  L  S  A  L
AGGAGAATAGCATGTGAAGAGGGCATTCGTGGGTTATACAGCGGTCTGGTGCCTGCTTTGGCTGGTGTTAGTCATGTAGCCATTCAGTTC
 R  R  I  A  C  E  E  G  I  R  G  L  Y  S  G  L  V  P  A  L  A  G  V  S  H  V  A  I  Q  F
CCAACTTATGAGAAAATTAAGATATACTTGGCCAAGCGAGATAACACTTCAACAGATAAACTCGGGGCCCCTGATGTTGCTGTTGCATCA
 P  T  Y  E  K  I  K  I  Y  L  A  K  R  D  N  T  S  T  D  K  L  G  A  P  D  V  A  V  A  S
TCAGTTTCCAAAATATTTGCTTCTACATTGACATATCCACATGAGGTAGTACGTTCAAGGCTTCAAGAACAGGGGCGCCATTCTGAAAAG
 S  V  S  K  I  F  A  S  T  L  T  Y  P  H  E  V  V  R  S  R  L  Q  E  Q  G  R  H  S  E  K
CGGTACTCTGGTGTAATTGACTGCATTAAAAAAGTGTTTCATCAAGAAGGCATCCAAGGCTTTTACCGAGGTTGTGCGACCAACCTAATT
 R  Y  S  G  V  I  D  C  I  K  K  V  F  H  Q  E  G  I  Q  G  F  Y  R  G  C  A  T  N  L  I
CGGACCACCCCTGCAGCTGTTATCACATTTACAAGTTTTGAGATGATTCACCGCTTCCTTGTAACCTTGTTTCCCGCTGATCCTCATCCT
 R  T  T  P  A  A  V  I  T  F  T  S  F  E  M  I  H  R  F  L  V  T  L  F  P  A  D  P  H  P
CACCCATTGTAGGATGTCATGACTTGGGGATTCACCATAGAGGCAGTTTAGTTCATCAAGTCTTTGAGCGAGGTGGCTAATTTGGCTGCAGC
 H  P  L  *
AATCTCCTCATTAGTTGTGTATCTTATTTTTTGTTAGCAACATAGAGAAGCCATCTTGGACATTGTGAGACAAATTTGCTATACCATTGTTA
CACTGAAATTGATGATCATGATGATGATAAAAGGTGTTGAAAGTAGGAACTAGGAAAACGAGATTATACGAGCTTTGACAAAATTCAGTATT
GAATGAAACTCTGTTGAGTGCAAAAAAAAAAAAAAAAAAAAAAAAAAA
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Fig. 2: The complete mRNA of tobacco nicotinamide adenine dinucleotide transporter 1 gene and its encoding amino acids. '*' indicates the stop codon

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Common tobacco          ----------------------MAADTHGPTPKGLLCNAGAGAAAGVIAAT
Lycopersicon esculentum ------------MTLQACEGDLAMAADRPTPKGVLCNAGAGAAAGVIAAT
Wine grape              ----------------------MPAESHAPSPRGLLCNAGAGAAAGVIAAT
Soybean                 -------------------MTADTHAAPNINPKGLLCNAAAGASAGVIAAT
Chickpea                --------------------MSTDSHAPNPYPRGLLCNAGAGAAAGVIAAT
Foxtail millet          MPGDVPTSPAPAPPSASSSSQHHHHRGPTSARGLLCHAVAGASAGVVAAT
                                                 .:*:**:* ***:***:***

Common tobacco          FVCPLDVIKTRLQVHGLPKIGTANVRGSLIVGSLEQIFQREGLRGMYRGL
Lycopersicon esculentum FVCPLDVIKTRLQVHGLPKLGNANVRGSLIVGNLEHIFQKEGLRGMYRGL
Wine grape              FVCPLDVIKTRFQVHGLPQLGNGNIKGSLIVGSLEQIFQKEGLRGMYRGL
Soybean                 FVCPLDVIKTRFQVHGVPQLAHRSAKGSIIVASLEQVFHKEGLRGMYRGL
Chickpea                FVCPLDVIKTRFQVHGSLQLANGTVKGSLIVGSLEQIYHKEGLRGMYRGL
Foxtail millet          FVCPLDVIKTRFQVHGWPKLGTGTIGGSVIIGSLQQIAQREGFRGMYRGL
                        ***********:****  ::.  .  **:*:..*::: ::**:*******

Common tobacco          SPTVLALLPNWAVYFTIYEQLKSFLGSDDGSHQLSIGANMLAASGAGAAT
Lycopersicon esculentum SPTVLALLPNWAVYFTIYDQLKRFLGSDDGSHQLTIGANMLAASGAGAAT
Wine grape              SPTVLALLPNWAVYFTIYEQLKSFLCSNDENHQLSIGANMIAACGAGAAT
Soybean                 APTVLALLPNWAVYFSAYEQLKSLLQSDD-SHHLSIGANMIAASGAGAAT
Chickpea                APTVLALLPNWAVYFTVYEQLKSLLCSDDKNHHLSVGANVIAASGAGAAT
Foxtail-millet          SPTVLALLPNWAVYFTVYEQLKSLLSSNDGSHQLSLGANVVAASCAGAAT
                        :***************: *:*** :* *:* .*:*::***::**. *****

Common tobacco          TIATNPLWVVKTRLQTQGMKAGLEPYRGTLSALRRIACEEGIRGLYSGLV
Lycopersicon esculentum TIATNPLWVVKTRLQTQGMRTGFEPYRGTLSALRRIAYEEGIRGLYSGLV
Wine grape              TIATNPLWVVKTRLQTQGMRAGVVPYSSTLSALRRIAYEEGIRGLYSGLV
Soybean                 TMFTNPLWVVKTRLQTQGMRPGVVPYRGTLSALRRIAHEEGIRGLYSGLV
Chickpea                TVVTNPFWVVKTRLQTQGMRPGVVPYRNTLSALKRIACEEGIRGMYSGLV
Foxtail millet          SIVTNPLWVVKTRFQTQGIRAGPIPYKGTLAALSRIAHEEGIRGLYSGLV
                        :: ***:******:****::.*  ** .**:** *** ******:*****

Common tobacco          PALAGVSHVAIQFPTYEKIKIYLAKRDNTSTDKLGAPDVAVASSVSKIFA
Lycopersicon-esculentum PALAGVSHVAIQFPTYEKIKIYLAKRANTSTDKLGAPDVAVASSVSKIFA
Wine grape              PALAGISHVAIQFPTYEKIKMYLASRENTTMDKLGAPDVAVASSVSKIFA
Soybean                 PALAGISHVAIQFPTYETIKFYLANQDDTAMEKLGARDVAIASSVSKIFA
Chickpea                PALAGISHVAIQFPTYERIKFHLANKDNTTVDKLGARDVAIASSVSKFFA
Foxtail millet          PALAGVSHVAIQFPAYEKIKAYLAERDNTTVEALSFGDVAVASSLAKVAA
                        *****:********:** ** :**.: :*: : *.  ***:***::*. *

Common tobacco          STLTYPHEVVRSRLQEQGRHSEKRYSGVIDCIKKVFHQEGIQGFYRGCAT
Lycopersicon esculentum STLTYPHEVVRSRLQEQGRHSEKRYSGVVDCIKKVFHQEGITGFYRGCAT
Wine grape              STLTYPHEVVRSRLQEQGHHSEKRYSGVVDCIKKVLQQEGLAGFYRGCAT
Soybean                 STLTYPHEVVRSRLQEQGHHSEKRYSGVIDCIRKVFHQEGVSGFYRGCAT
Chickpea                STLTYPHEVVRARLQEQGHHSEKRYSGVSDCIRKVFQQEGLSGFYRGCAI
Foxtail millet          STMTYPHEVVRSRLQDQGAHSETRYKGVIDCIKKVYYKEGVAGFYRGCAT
                        **:*********:***:** ***.**.** ***:**  :**: *******

Common tobacco          NLIRTTPAAVITFTSFEMIHRFLVTLFPADPHPHPL----
Lycopersicon esculentum NLIRTTPAAVITFTSFEMIHRFLVTMFPPDPHPHTL----
Wine grape              NLLRTTPAAVITFTSFEMIHRFLVNLLPPDPHPHTL----
Soybean                 NLLRTTPAAVITFTSFEMIHRFLVSYFPSDPRPHIL----
Chickpea                NLLRTTPAAVITFTSFEMIQRFLVSLSPSDQQPHNL----
Foxtail millet          NLLRTTPAAVITFTSFEMIHRFLLDLIPPEPEPHIQPLKH
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Fig. 3: The alignment of the proteins encoded by tobacco and other nicotinamide adenine dinucleotide transporter 1 genes

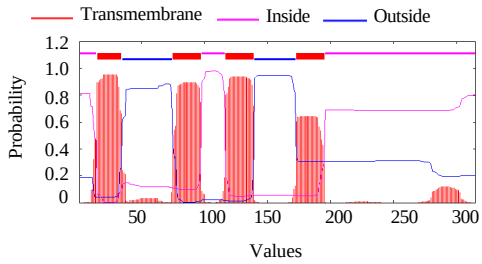

Fig. 4: The transmembrane protein prediction of tobacco nicotinamide adenine dinucleotide transporter 1 protein

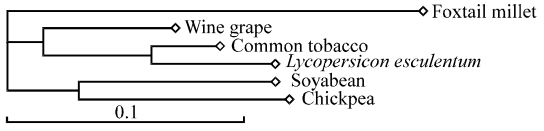

Fig. 5: The phylogenetic tree for five kinds of nicotinamide adenine dinucleotide transporter 1 genes

the tobacco nicotinamide adenine dinucleotide transporter 1 gene or use tobacco as model organism to study the lycopersicon esculentum nicotinamide adenine dinucleotide transporter 1 gene.

From the tissue distribution analysis in the experiment, it can be seen that nicotinamide adenine dinucleotide transporter 1 gene was highly expressed in leaf. For nicotinamide adenine dinucleotide transporter 1 has been characterized to be responsible for this transport activity (Todisco *et al*., 2006). The suitable explanation for this under current conditions is that nicotinamide adenine dinucleotide transport is mainly existed in leaf.

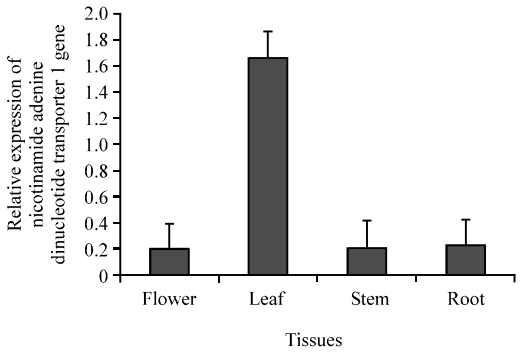

Fig. 6: Expression analysis of nicotinamide adenine dinucleotide transporter 1 gene *mRNA* in various tissues

CONCLUSION

Researchers first isolated the tobacco nicotinamide adenine dinucleotide transporter 1 gene and performed necessary sequence analysis and tissue expression profile analysis. These will establish the primary foundation of using tobacco nicotinamide adenine dinucleotide as drug for animals and humans in the future.

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