

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

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Abstract: Nicotinamide adenine dinucleotide have significant health benefits for animals and humans. Peroxisomal nicotinamide adenine dinucleotide carrier gene functions in mediating the nicotinamide adenine dinucleotide import into peroxisomes. The complete coding sequence of tobacco peroxisomal nicotinamide adenine dinucleotide carrier gene was amplified by RT-PCR. The open reading frame of tobacco peroxisomal nicotinamide adenine dinucleotide carrier gene was 1017 bp which encodes a protein of 338 amino acids. Sequence analysis revealed that the peroxisomal nicotinamide adenine dinucleotide carrier of tobacco shares high homology with the peroxisomal nicotinamide adenine dinucleotide carrier of *Lycopersicon esculentum* (88%), *Fragaria vesca* subsp. *vesca* (74%), thale cress (73%), chickpea (73%) and foxtail millet (67%). Phylogenetic tree analysis revealed that the tobacco peroxisomal nicotinamide adenine dinucleotide carrier gene has a closer genetic relationship with that of *Lycopersicon esculentum*. Prediction of transmembrane helices showed that tobacco peroxisomal nicotinamide adenine dinucleotide carrier might be a transmembrane protein. Expression profile was studied and the results indicated that tobacco peroxisomal nicotinamide adenine dinucleotide carrier gene was highly expressed in stem, leaf and flower. These results established the primary foundation of using tobacco nicotinamide adenine dinucleotide as drug for animals and humans in the future.

Key words: Tobacco, gene, peroxisomal nicotinamide adenine dinucleotide carrier, expression pattern, millet

INTRODUCTION

Recent evidences indicated 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). 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 Lidup, 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).

Based on the described 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 peroxisome and must be imported across the permeability barrier of the inner peroxisome membrane. Peroxisomal nicotinamide adenine dinucleotide carrier has been characterized to be responsible for this transport function. Peroxisomal nicotinamide adenine dinucleotide carrier mediates the nicotinamide adenine dinucleotide import into peroxisomes. It favors the nicotinamide adenine dinucleotide (in)/AMP (out) antiport exchange and is required for peroxisomes proliferation (Mano *et al.*, 2011; Agrimi *et al.*, 2012; Bernhardt *et al.*, 2012).

Peroxisomal nicotinamide adenine dinucleotide carrier gene has been identified from many plants. Until today, the tobacco peroxisomal nicotinamide adenine dinucleotide carrier 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.

Table 1: PCR primers for tobacco peroxisomal nicotinamide adenine dinucleotide carrier gene isolation

| Genes | Primer sequence | Ta (°C) | Length (bp) |
|---|-------------------------------------|---------|-------------|
| Peroxisomal nicotinamide adenine dinucleotide carrier | Forward : 5'-ATGTCGGACGCTTTGATC-3' | 56 | 1017 |
| | Reverse: 5'-CTAATGAGGCTTTAATCTGA-3' | | |

Table 2: qRT-PCR primers for tobacco peroxisomal nicotinamide adenine dinucleotide carrier, actin genes and annealing temperature

| Genes | Primer sequence | Ta (°C) | Length (bp) |
|---|------------------------------------|---------|-------------|
| Peroxisomal nicotinamide adenine dinucleotide carrier | Forward: 5'-GCGTCGCAGGGTGTAT-3' | 58 | 285 |
| | Reverse: 5'-CCCATAGGGAGGAGGTTTC-3' | | |
| Actin | Forward :5'-CCATTCCTCGTTGGACCTT-3' | 56 | 257 |
| | Reverse: 5'-TTCTGGGCAACGGAACCT-3' | | |

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 peroxisomal nicotinamide adenine dinucleotide carrier 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 DNA polymerase buffer, 1.2 µL 25 mM MgCl₂, 1.0 µL 10 mM forward primer, 1.0 µL 10 mM reverse primer, 2.0 units of Taq DNA polymerase (1U/1 µL) and 9.8 µL sterile water. The primers for tobacco peroxisomal nicotinamide adenine dinucleotide carrier gene isolation were designed based on the tobacco EST sequences (GeneBank numbers FG625827 and FG157895) which are highly homologues with the coding sequence of peroxisomal nicotinamide adenine dinucleotide carrier 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 L min⁻¹, 56°C (Table 1)/1 min, 72°C L min⁻¹ 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: qRT-PCR for evaluating the level of mRNA for peroxisomal nicotinamide adenine dinucleotide carrier 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 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^{-ΔΔCt} 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. 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

Isolation result for tobacco peroxisomal nicotinamide adenine dinucleotide carrier gene: For tobacco peroxisomal nicotinamide adenine dinucleotide carrier gene, through RT-PCR with pooled tissue cDNAs, the resulting PCR products were 1017 bp (Fig. 1).

Sequence analysis: The cDNA nucleotide sequence analysis using the BLAST Software at NCBI server 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.: KF856281).

The sequence prediction showed that the 1017 bp cDNA sequence represents one single gene which encodes 338 amino acids (Fig. 2). 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. The pI of tobacco peroxisomal nicotinamide adenine dinucleotide carrier is 9.75. The molecular weight of this putative

protein is 37051.98. Further BLAST analysis of these proteins revealed that tobacco peroxisomal nicotinamide adenine dinucleotide carrier has high homology with the peroxisomal nicotinamide adenine dinucleotide carrier of *Lycopersicon esculentum* (Accession No.: XP_004239938, 88%), *Fragaria vesca* subsp. *vesca* (Accession No.:XP_004307616, 74%), thale cress (Accession No.: NP_181526, 73%), chickpea (Accession No.: XP_004490955, 73%) and foxtail millet (Accession No.: XP_004984687, 67%) (Fig. 3).

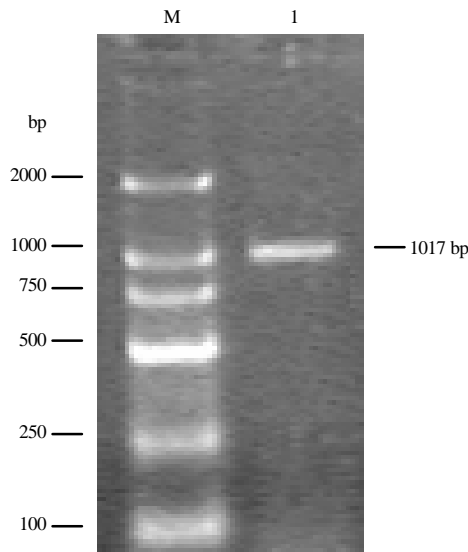


Fig. 1: PCR result for tobacco peroxisomal nicotinamide adenine dinucleotide carrier gene. M: DL2000 DNA markers; 1: PCR product for tobacco peroxisomal nicotinamide adenine dinucleotide carrier gene

Based on the results of the alignment of different peroxisomal nicotinamide adenine dinucleotide carrier proteins, a phylogenetic tree was constructed using the ClustalW Software as shown in Fig. 4. The phylogenetic analysis revealed that the tobacco peroxisomal nicotinamide adenine dinucleotide carrier gene has a closer genetic relationship with that of *Lycopersicon esculentum*.

The prediction of transmembrane helices in protein using the TMHMM Server V. 2.0 showed that tobacco peroxisomal nicotinamide adenine dinucleotide carrier protein might be a transmembrane protein (Fig. 5).

Tissue expression profile: Tissue expression profile analysis was carried out and results revealed that the tobacco peroxisomal nicotinamide adenine dinucleotide carrier gene was highly expressed in stem, leaf and flower but hardly expressed in root (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 peroxisomal nicotinamide adenine dinucleotide carrier genes, it can be seen that the coding sequences of peroxisomal nicotinamide adenine dinucleotide carrier genes were highly conserved in two solanaceae plants-tobacco and *Lycopersicon esculentum*, but not conserved in other plants.

The phylogenetic tree analysis revealed that the tobacco peroxisomal nicotinamide adenine dinucleotide

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ATGTCGGACGCTTTGATCAGTGGCCTCGCCGGTGGCCGGCGGGCATCATCGCTCAGCTCATCACCTATCCTCTACAACAGTAAATACT
M S D A L I S G L A G A G G G I I A Q L I T Y P L Q T V N T
CGGCAGCAACGGATAGAAATCTAAGAAAATGAAGCAACGCTTGGAAAGTATCGAACAAATTTGCCAGGTTGTAAAGCAAGAAGGATGG
R Q Q T D R N S K K M K Q T L G S I E Q I C Q V V K Q E G W
GATGGGCTATATGGTGGCTTAACCTCCGCTCGCTAATTTGGTACCGCTGCGTCCGAGGGTGTATTATTATTCTACCAAAATATTCAGGAAT
D G L Y G G L T P S L I G T A A S Q G V Y Y Y F Y Q I F R N
AGAGCAGAACTGATGCCCTTGAACGTAAGAAAAAAGGAATTTGGTATGGATCAGTGGGAATGTTCTCTTCATCTACTGGTGGCTGCTTTA
R A E T D A L E R K K K G I G D G S V G M F S S L L V A A L
TCTGGGTGTGTTAACGTGCTGCTGACCAATCCCATATGGGTAGTTGTACTCGCATGCAGACTCATACAAGAAAAGATAAAGATAACCAC
S G C V N V L L T N P I W V V V T R M Q T H T R K D K D N H
ACCAAACCTGTAACATCACAGGATGCAATATCTGCTGTGGTTGAACCTCCTCCCTATGGGACAAGCTACACTATTCAGGAAGTCTACGGT
T K F V T S Q D A I S A V V E P P P Y G T S Y T I Q E V Y G
GAAGCTGGAGITTTGGGGTTTCTGGAATGGTGTITTTCCAACATTATTATGGTAAAGCAATCCTTCCATACAATTTATGCTATATGAAACC
E A G V W G F W N G V F P T L F M V S N P S I Q F M L Y E T
TTGCTGAAGAAGATCAGGAAACGACGTGCCCTTAGCAACAAAGGTGCCAATGATGTCACCTGCTTTGGAGATATTTCTACTCGGAGCTGTA
L L K K I R K R R A S S N K G A N D V T A L E I F L L G A V
GCAAAACTTGGAGCTACGGTTGTAACGTATCCCTTCTGGTTATCAAGTCCAGCTTCAGGCAAGCAGGATGTTGGTGGGGATAAAGA
A K L G A T V V T Y P L L V I K S R L Q A K Q D V G G D K R
CATCACTATAAAGCACACTTACGCTATAGTGAAGATTTACGCTATGAAGGCTTTCATGGATTGCAAGGGATGAGCACAAAATT
H H Y K G T L D A I V K I L R Y E G F H G F C K G M S T K I
GTACAAAGTGTCTTCTGCTGCTGTTCTATTATCGGTCAAGGAAGAACTGTGAGGGGTGCTAGATGGTTATTAACAGGTGCTGCTGCT
V Q S V L A A A V L F M V K E E L V R G A R W L L T G R S A
ACTTCTGCAGATTAAGCCTCATTAG
T S V R L K P H *
    
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Fig. 2: The complete coding sequence of tobacco peroxisomal nicotinamide adenine dinucleotide carrier gene and its encoding amino acids. ‘*’ indicates the stop codon

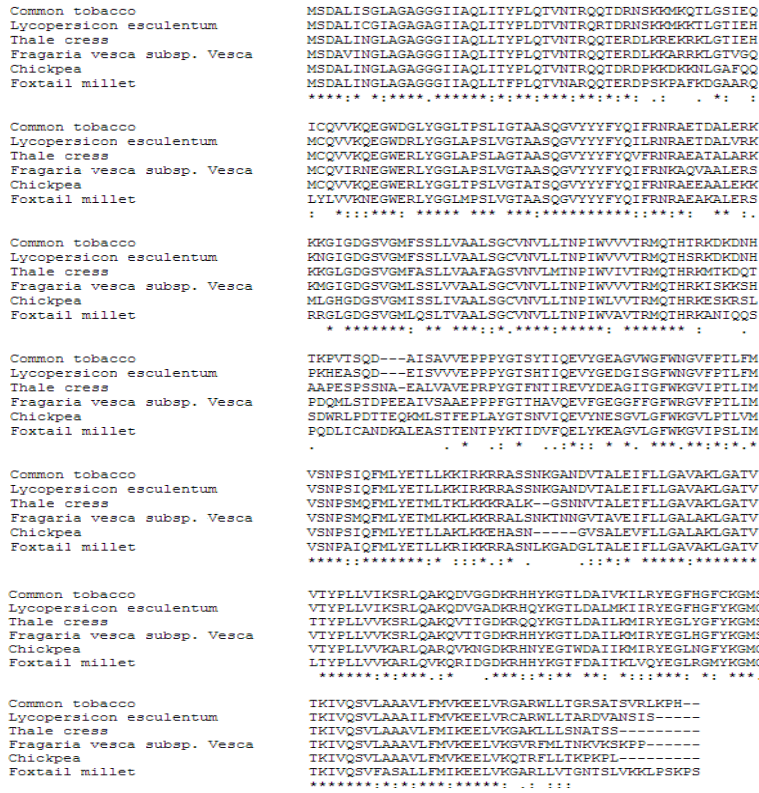


Fig. 3: The alignment of the proteins encoded by tobacco and other peroxisomal nicotinamide adenine dinucleotide carrier genes

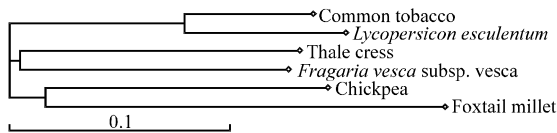


Fig. 4: The phylogenetic tree for five kinds of peroxisomal nicotinamide adenine dinucleotide carrier genes

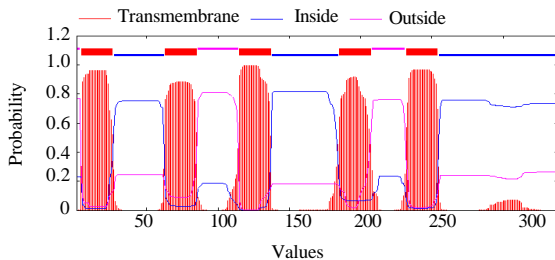


Fig. 5: The transmembrane protein prediction of tobacco peroxisomal nicotinamide adenine dinucleotide carrier protein

carrier gene has a closer genetic relationship with that of *Lycopersicon esculentum*. This implied that researchers

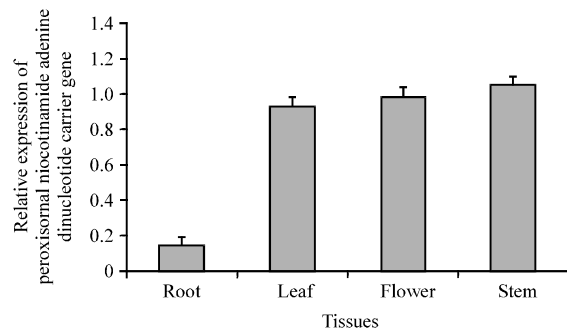


Fig. 6: Expression analysis of peroxisomal nicotinamide adenine dinucleotide carrier gene mRNA in various tissues

can use *Lycopersicon esculentum* as model organism to study the tobacco peroxisomal nicotinamide adenine dinucleotide carrier gene or use tobacco as model organism to study the *Lycopersicon esculentum* peroxisomal nicotinamide adenine dinucleotide carrier gene.

From the tissue distribution analysis in the experiment it can be seen that peroxisomal nicotinamide

adenine dinucleotide carrier gene was highly expressed in stem, leaf and flower. For peroxisomal nicotinamide adenine dinucleotide carrier had been characterized to be required for nicotinamide adenine dinucleotide import into peroxisomes, the suitable explanation for this under current conditions is that this transport function is mainly in stem, leaf and flower.

CONCLUSION

Researchers first isolated the tobacco peroxisomal nicotinamide adenine dinucleotide carrier gene and performed necessary sequence analysis and tissue expression profile analysis. These results established the primary foundation of using tobacco nicotinamide adenine dinucleotide as drug for animals and humans in the future.

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