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Molecular Characterization and Expression Pattern of a Novel Cadmium/Zinc-Transporting *ATPase HMA1* Gene

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Abstract: Cadmium (Cd) of tobacco is a pollutant that is extremely toxic to the health of humans. Cadmium/zinc-transporting *ATPase HMA1* gene has been characterized to function in the plant cadmium/zinc-transporting. The complete coding sequence of tobacco cadmium/zinc-transporting *ATPase HMA1* gene was amplified by RT-PCR. The open reading frame of tobacco cadmium/zinc-transporting *ATPase HMA1* gene was 2418 bp which encodes a protein of 805 amino acids. BLAST analysis revealed that tobacco cadmium/zinc-transporting ATPase HMA1 protein shares high homology with the cadmium/zinc-transporting ATPase HMA1 of potato (85%), *Lycopersicon esculentum* (84%), wine grape (71%), sweet orange (71%), soybean (69%) and thale cress (68%). Results also showed that tobacco cadmium/zinc-transporting *ATPase HMA1* gene has a closer genetic relationship with the cadmium/zinc-transporting *ATPase HMA1* gene of *Lycopersicon esculentum*. Prediction of transmembrane helices showed that tobacco cadmium/zinc-transporting ATPase HMA1 might be a transmembrane protein. The expression profile was studied and results indicated that tobacco cadmium/zinc-transporting *ATPase HMA1* gene was moderately expressed in root, leaf and stem but hardly expressed in flower. These results established the primary foundation of utilizing tobacco cadmium/zinc-transporting *ATPase HMA1* gene to descrease the cadmium content of tobacco and benefit the health of humans in the future.

Key words: Tobacco, gene, cadmium/zinc-transporting ATPase HMA1, expression pattern, established

INTRODUCTION

Cadmium (Cd) of tobacco is a pollutant that is extremely toxic to the health of humans. Cd has caused neurotoxicologic and behavioral changes in both humans and experimental animal studies (Liu *et al*., 2013; Counter *et al*., 2009). Cd exposure may be implicated in some neurological disorders including hyperactivity and increased aggressiveness in human (Liu *et al*., 2013; Maes *et al*., 2010). In the case of coronary risk with metal levels, Cd may be more important for females (Liu *et al*., 2013; Olsen *et al*., 2012). Cd was reported to damage bone microstructure and can negatively influence growth in newborns (Liu *et al*., 2013; Chen *et al*., 2011). Several studies have reported an inverse relationship between anthropometric measurements of the newborn and the placental or umbilical cord Cd level (Liu *et al*., 2013; Llanos and Ronco, 2009; Ronco *et al*., 2009). Cd exposure exerts inhibitory effects on testicular steroidogenesis (Liu *et al*., 2013; Pillai *et al*., 2012).

Cadmium/zinc-transporting ATPase HMA1 is a member of P (IB)-ATPase family that is localized to the chloroplast envelope and is involved in the plant transport of cadmium, zinc, copper and cobalt (Moreno *et al*., 2008; Kim *et al*., 2009; Higuchi *et al*., 2009). It is essential for growth under high light conditions (Seigneurin-Berny *et al*., 2006). Cadmium/zinc-transporting *ATPase HMA1* gene has been identified from many plants such as thale cress, tomato and potato. Until today, the tobacco cadmium/zinc-transporting *ATPase HMA1* gene has not been reported yet. In present experiment, researchers will isolate the coding sequence 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 cadmium/zinc-transporting *ATPase HMA1* gene to descrease the cadmium content of tobacco and benefit the health of humans in the future.

MATERIALS AND METHODS

Samples collection, RNA extraction and first-strand cDNA synthesis: The tissues including leave, stem, root, flower from tobacco plants (Chinese local variety Yunyan 87) in the stage of anthesis 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).

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Isolation of the coding sequence: RT-PCR was performed to amplify the coding sequence of tobacco cadmium/zinc-transporting *ATPase HMA1* 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 $MgCl_2$, 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 cadmium/zinc-transporting *ATPase HMA1* gene isolation were designed based on the tobacco EST sequences (GeneBank numbers FG145467 and AM812255) which are highly homologues with the coding sequence of cadmium/zinc-transporting *ATPase HMA1* 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/50 sec, 55°C (Table 1) 50 sec, 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: qRT-PCR for evaluating the level of mRNA for cadmium/zinc-transporting *ATPase HMA1* 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 $2^{-\Delta\Delta C}{}_T$ value model (Livak and Schmittgen, 2001).

Sequence analysis: mRNA sequence prediction was conducted using GenScan Software (http://genes.mit.edu/GENSCAN.html). Protein conserved domain analysis was carried out by conserved domain architecture retrieval tool of BLAST at the National Center for Biotechnology Information (NCBI) server (http://www.ncbi.nlm.nih.gov/BLAST). Theoretical isoelectric point (pI) and Molecular weight (Mw) of the deduced protein were computed using the Compute pI/Mw Tool (http://www.expasy.org/tools/pi_tool.html). Protein alignment analysis and phylogenetic tree analysis were performed with Clustalw Software (http://www.ebi.ac.uk/clustalw).

RESULTS AND DISCUSSION

Isolation result for tobacco cadmium/zinc-transporting *ATPase HMA1* gene: For tobacco cadmium/zinc-transporting *ATPase HMA1* gene, through RT-PCR with pooled tissue cDNAs, the resulting PCR products were 2418 bp (Fig. 1).

Sequence analysis: BLAST analysis of this cDNA 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 No.: KJ159917).

The sequence prediction was carried out using the GenScan Software and results showed that the 2418 bp cDNA sequence represents one single gene which

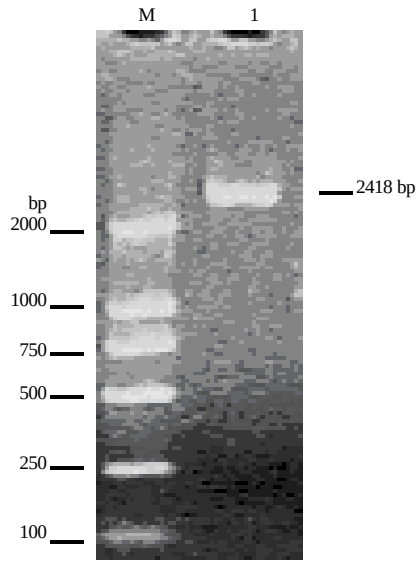

Fig. 1: PCR result for tobacco cadmium/zinc-transporting ATPase HMA1 gene; M: DL2000 DNA markers; 1: PCR product for tobacco cadmium/zinc-transporting *ATPase HMA1* gene

Table 1: PCR primers for tobacco cadmium/zinc-transporting *ATPase HMA1* gene isolation

Genes	Primer sequence	Ta (°C)	Length (bp)
Cadmium/zinc-transporting *ATPase HMA1*	Forward: 5'-ATGGAAGCTCTGCGTCTT-3' Reverse: 5-TCACAAATGGGCTGCTTG-3'	55	2418

Table 2: qRT-PCR primers for tobacco cadmium/zinc-transporting *ATPase HMA1*, *actin* genes and annealing temperatures

Genes	Primer sequence	Ta (°C)	Length (bp)
Cadmium/zinc-transporting *ATPase HMA1*	Forward: 5'-CTGCGGCTTTGTTTATTG-3' Reverse: 5'-GAGCCAACTTCCAGGTCA-3'	53	418
Actin	Forward: 5'-CCATTCTTCGTTTGGACCTT-3' Reverse: 5'-TTCTGGGCAACGGAACCT-3'	56	257

encodes a protein of 805 amino acids (Fig. 2). The theoretical isoelectric point (pI) and Molecular weight (Mw) of this deduced protein were also computed. The pI is 8.27. The molecular weight of this putative protein is 87498.28 Da.

Further, BLAST analysis of this protein revealed that it shares high homology with the cadmium/zinc-transporting ATPase HMA1 of potato (Accession No: XP_006339845, 85%), *Lycopersicon esculentum* (Accession No: XP_004231883, 84%), wine grape (Accession No: XP_002278549, 71%), sweet orange (Accession No: XP_006466544, 71%), soybean (Accession No: XP_003550994, 69%) and thale cress (Accession No: NP_195444, 68%) (Fig. 3).

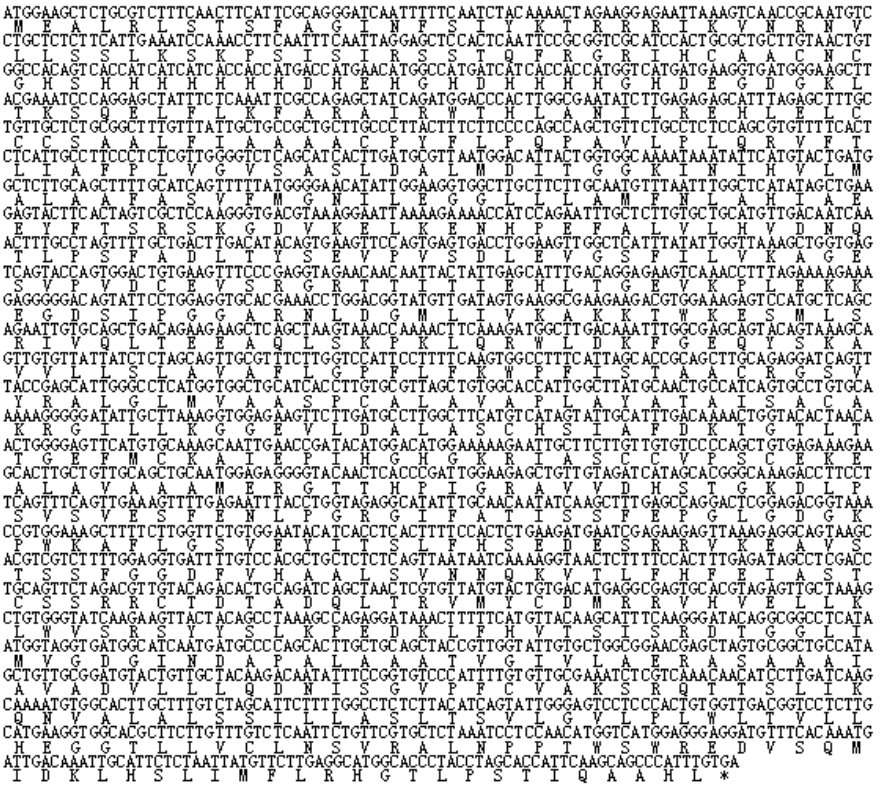

Fig. 2: The complete coding sequence of tobacco cadmium/zinc-transporting *ATPase HMA1* gene and its encoding amino acids; *indicates the stop codon

Fig. 3: Continue

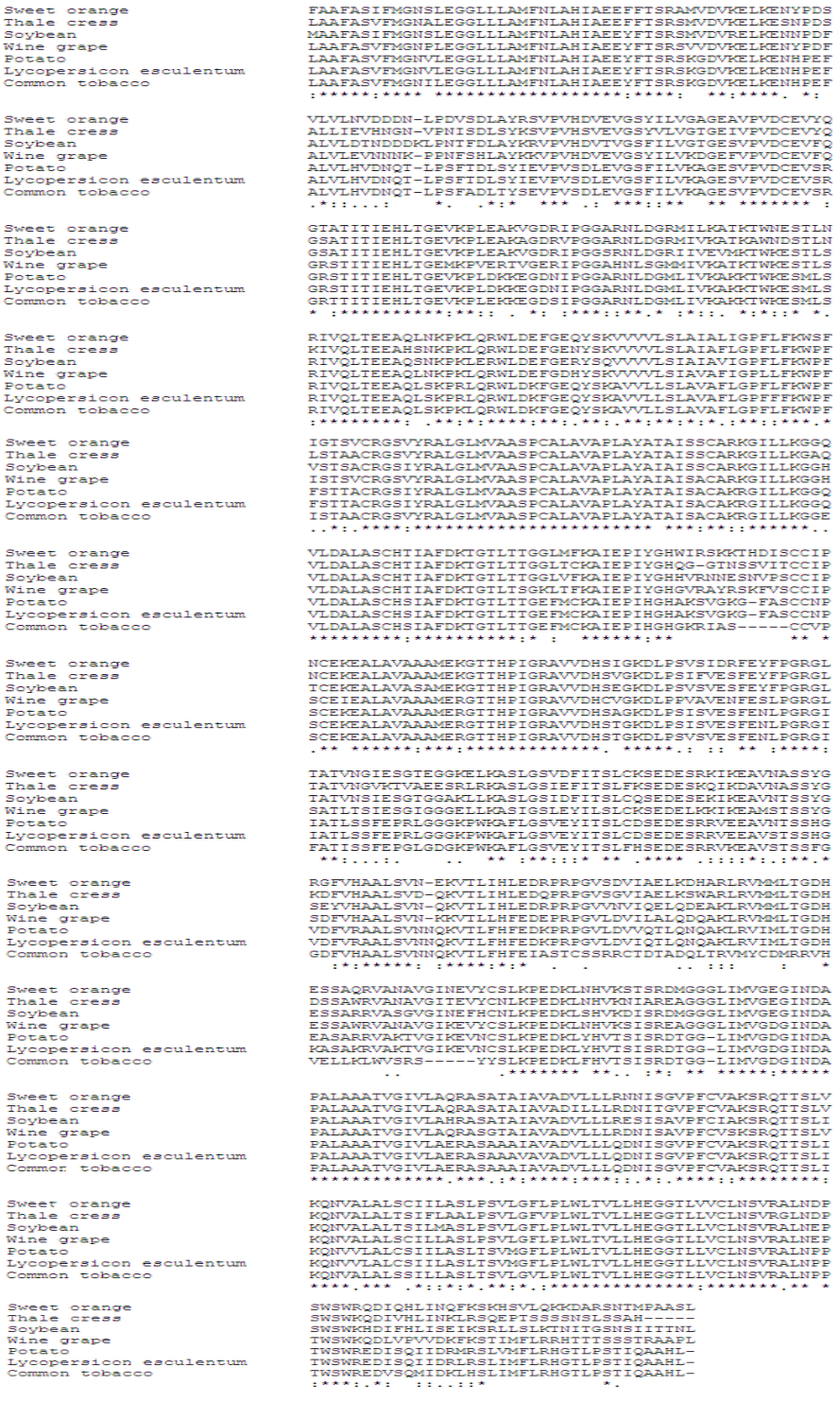

Fig. 3: The alignment of cadmium/zinc-transporting ATPase HMA1 proteins

From the results obtained above, it can be concluded that this protein is the tobacco cadmium/zinc-transporting ATPase HMA1 and the new gene is the tobacco cadmium/zinc-transporting *ATPase HMA1* gene. Based on the results of the alignment of different species of cadmium/zinc-transporting ATPase HMA1 proteins, a

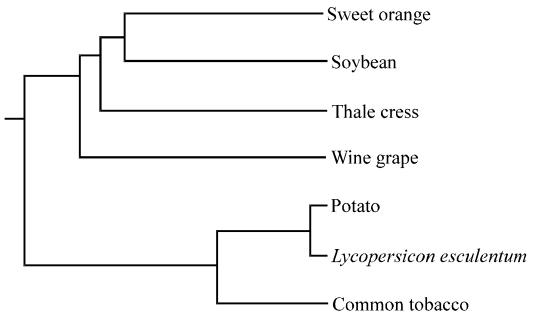

Fig. 4: The phylogenetic tree for seven kinds of cadmium/zinc-transporting *ATPase HMA1* genes

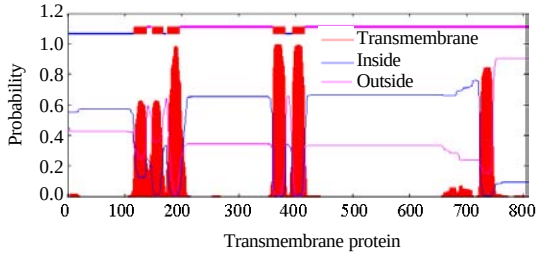

Fig. 5: The transmembrane protein prediction of tobacco cadmium/zinc-transporting ATPase HMA1

phylogenetic tree was constructed using the ClustalW Software as shown in Fig. 4. Phylogenetic analysis revealed that the tobacco cadmium/zinc-transporting *ATPase HMA1* 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 (http://www.cbs.dtu.dk/services/TMHMM/) showed that tobacco cadmium/zinc-transporting ATPase HMA1 might be a transmembrane protein (Fig. 5).

Tissue expression profile: Tissue expression profile analysis was carried out and results revealed that the tobacco cadmium/zinc-transporting *ATPase HMA1* gene was moderately expressed in root, leaf and stem but hardly expressed in flower (Fig. 6).

Modern comparative genomics research has revealed that virtually all (99%) of the protein-coding genes of humans share high homology with the that of mouse 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 cadmium/zinc-transporting *ATPase HMA1* genes, it can be seen that the coding sequences of cadmium/zinc-transporting *ATPase HMA1* genes were highly conserved in three solanaceae plants-tobacco, potato and *Lycopersicon esculentum*.

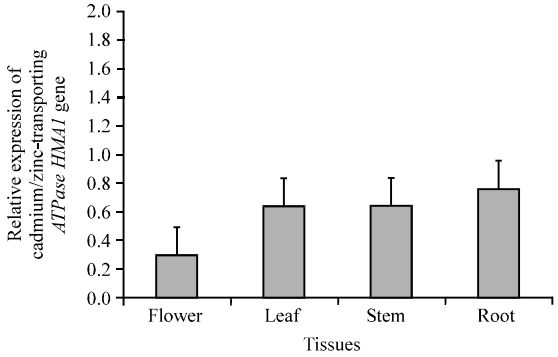

Fig. 6: Expression analysis of cadmium/zinc-transporting *ATPase HMA1* gene mRNA in various tobacco tissues

The phylogenetic tree analysis revealed that the tobacco cadmium/zinc-transporting *ATPase HMA1* 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 cadmium/zinc-transporting *ATPase HMA1* gene or use tobacco as model organism to study the *Lycopersicon esculentum* cadmium/zinc-transporting *ATPase HMA1* gene.

From the tissue distribution analysis in the experiment it can be seen that cadmium/zinc-transporting *ATPase HMA1* gene was moderately expressed in root, leaf and stem and hardly expressed in flower. For cadmium/zinc-transporting ATPase HMA1 functions in the transport of cadmium, zinc, copper and cobalt (Moreno *et al*., 2008; Kim *et al*., 2009; Higuchi *et al*., 2009), the suitable explanation for this is that the transport process of cadmium, zinc, copper and cobalt is mainly existed in root, leaf and stem in the stage of anthesis. These merit further study.

CONCLUSION

Researchers first isolated the tobacco cadmium/zinc-transporting *ATPase HMA1* gene. These will establish the primary foundation of utilizing tobacco cadmium/zinc-transporting *ATPase HMA1* gene to descrease the cadmium content of tobacco and benefit the health of humans in the future.

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