Original Article

MOLECULAR CHARACTERIZATION AND EXPRESSION PATTERN OF A NOVEL CADMIUM RESISTANCE GENE OF TOBACCO

CARACTERIZAÇÃO E PADRÃO DE EXPRESSÃO MOLECULAR DE UM NOVO GENE DE FUMO PARA RESISTÊNCIA AO CÁDMIO

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ABSTRACT: Cadmium (Cd) of tobacco is a pollutant that is extremely toxic to the health of humans. Protein plant cadmium resistance 8 gene has been characterized to increase the plant Cd resistance. In present experiment, the complete mRNA sequence of tobacco protein plant cadmium resistance 8 gene was amplified using the rapid amplification of cDNA ends methods. The full-length tobacco protein plant cadmium resistance 8 gene mRNA was 887bp containing an 555 bp open reading frame, which encodes a protein of 184 amino acids. BLAST analysis revealed that tobacco protein plant cadmium resistance 8 protein shares high homology with the protein plant cadmium resistance 8 of potato (81%), *Lycopersicon esculentum* (80%), *Eutrema salsugineum* (60%), *Capsella rubella* (58%) and thale cress (57%). Results also showed that tobacco protein plant cadmium resistance 8 gene has a closer genetic relationship with the protein plant cadmium resistance 8 gene of *Lycopersicon esculentum*. The expression profile was studied and the results indicated that tobacco protein plant cadmium resistance 8 gene was highly expressed in root, moderately expressed in stem, and hardly expressed in flower and leaf. These results established the primary foundation of utilizing tobacco protein plant cadmium resistance 8 gene to decrease the cadmium content of tobacco and benefit the health of humans in the future.

KEYWORDS: Tobacco. Gene. Protein plant cadmium resistance 8. Expression pattern.

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 humans (Liu et al., 2013; Matés 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 microstructures (Liu et al., 2013; Chen et al., 2011) and can negatively influence growth in newborns. Several studies have reported an inverse relationship between anthropometric measurements of the newborn and the placental or umbilical cord Cd levels (Liu et al., 2013; Llanos et al., 2009; Ronco et al., 2009). Cd exposure exerts inhibitory effects on testicular steroidogenesis(Liu et al., 2013; Pillai et al., 2012).

Recent researches have proved that a lot of genes, including protein plant cadmium resistance 8, can increase the plant Cd resistance (Thomine et al., 2000; Song et al., 2004; Ishikawa et al., 2012). Protein plant cadmium resistance 8 gene has been identified from many plants such as thale cress, tomato and potato. Until today, the tobacco protein plant cadmium resistance 8 gene has not been reported yet.

In the present work, we isolated the full-length mRNA sequence of this tobacco gene, subsequently performed some necessary sequence analysis and tissue expression analysis for this gene. These will establish the primary foundation of utilizing tobacco protein plant cadmium resistance 8 gene to decrease the cadmium content of tobacco and benefit the health of humans in the future.

MATERIAL AND METHODS

Samples collection, RNA extraction and first-strand cDNA synthesis

Tobacco plants (Chinese local variety Yunyan 87) were grown in a naturally lit glasshouse with normal irrigation and fertilization. The tissues including leave, stem, root and flower were harvested, immediately frozen in liquid nitrogen and stored at -80℃. 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 protein plant cadmium resistance 8 gene, the gene specific primers (GSPs) were designed based on one tobacco EST sequence: EH623456. 5'-RACE GSP: 5'-GCCCATTATCCATTGAGAGCAAACA-3' 3'-

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RACE GSP: 5'-GCCTGCTGTTTGCTCTCAATGGATA-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, 70°C 30 sec and 72 °C 3 min, finally with 25 cycles of 94°C 30 sec, 66°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 protein plant cadmium resistance 8gene was performed by the ABI Prism 7300 Sequence Detection Systems (Applied Biosystems, Foster City, CA, USA). 25µl reaction volume of PCR reaction contained 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, optimal annealing temperature for each specific primer for 15 s (Table 1), 72°C for 20 s. 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 et al., 2001).

Table 1. qRT-PCR primers for tobacco protein plant cadmium resistance 8 and actin genes

Gene	*Primer sequence*	*Ta/ °C*	*Length/(bp)*
protein plant cadmium resistance 8	Forward : 5'-TGGATACAATGGGAAGAG -3' Reverse: 5'- ATGGACAGAATATGTGGG -3'	52	420
Actin	Forward :5'-CCATTCTTCGTTTGGACCTT -3' Reverse: 5'- TTCTGGGCAACGGAACCT-3'	56	257

Sequence analysis

Gene prediction of cDNA sequence was performed by GenScan software (http://genes.mit.edu/GENSCAN.html). 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). 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).

RESULTS

RACE results for tobacco protein plant cadmium resistance 8 gene

For tobacco protein plant cadmium resistance 8 gene, through 5'-RACE, one PCR product of 380-bp was obtained. The 3'-RACE product was 538-bp in length. These products were then cloned to T-vector and sequenced. Taken together, a 887-bp cDNA sequence was finally obtained (Figure 1).

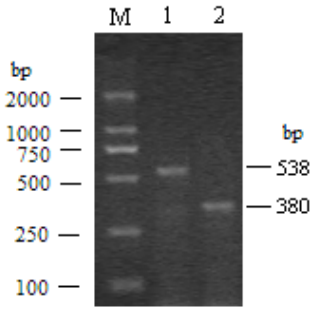

Figure1. RACE results for tobacco protein plant cadmium resistance 8 gene. M DL2000 DNA markers; 1, 3'-RACE product for tobacco protein plant cadmium resistance 8 gene; 2 , 5'-RACE product for tobacco protein plant cadmium resistance 8 gene

Sequence analysis

BLAST analysis of this cDNA sequence revealed that this sequence was not homologous to any of the known tobacco gene and it was then deposited into the Genbank database (Accession number: KJ159912).

Sequence prediction was carried out and results showed that this 887-bp cDNA sequence represents one single gene which encodes 184 amino acids(Figure 2). The theoretical isoelectric point (pI) and molecular weight (Mw) of this deduced protein was also computed. The pI of tobacco protein plant cadmium resistance 8 is 5.29. The molecular weight is 20371.27.

AATTTTCTTCATCCACTTGAGGTGTATACGTAGGTGTAAAATTTTTGGATACAATGGGAAGAGTTG
A A G A A A A A T A A T G A A A T A G A A A C T C C T
M G R V E E N N E I E T P
AAACCAGGTGAGAGTGGTGAACCAGTTGCCTCACAGCCTCCTCCACAGTACCAAGGAGTAAAGG
A T G T A C A G C C G C C A T C G C C A T C A C A A
K P G E S G E P V A S Q P P P Q Y Q G V K D V Q P P S P S Q
CCAATTGGAGCTCCCTGGAGCACTGGCTTATTTGATTGTCATTTGAACCAGATTAATGCTGTTATG
A C A T C A T T C T T A C C T T G T G T A A C A
P I G A P W S T G L F D C H L N Q I N A V M T S F L P C V T
TTCGGACAGATAGCAGAAGTCCTCGATGCAGGAGAAATGACATGTCCTTTGGGGACTTTCATATA
C T T G C T G A T G A T G C C T G C T G T T T G C
F G Q I A E V L D A G E M T C P L G T F I Y L L M M P A V C
TCTCAATGGATAATGGGCTCTAAGTATAGAACTAAGCTGAGACAGAAATATAATCTTGTCGAAGC
T C C T T A T T C A G A C A T A G T T C C C A C
S Q W I M G S K Y R T K L R Q K Y N L V E A P Y S D I V S H
ATATTCTGTCCATGTTGCTCTCTTTGTCAAGAGTTCAGAGAACTTCAACACAGGGGACTTGATCCT
G C T C T A G G A T G G A A T G G T A T A G T T
I F C P C C S L C Q E F R E L Q H R G L D P A L G W N G I V
GCTCAGCAGCATTATGGGAACCAACAAGTGAATCAAGCTCCCCAAGTACAATCCATGTCTAAGTA
A A A T T C A A G A T T C T G A T T C T T T A T T T G
A Q Q H Y G N Q Q V N Q A P Q V Q S M S K *
TTTGAGTTAAGGTTGTAATATGTTACATGTGTTTTGACACACCCAGATTCTGTTCATGTTGTAGCCT
TTATTATTTATTCCAACAATCTTGTTGGCAGTGTTGATTGTTGTACTATGTTTCTAGAACTCTATTTT
TGGGAGTTACATACTTCCGCGTATATGAAAATTGAGTTTGCAATGATCTAATGAAGTCTTGGCATC
AATTATTAATTGGGCAGTTCGATAAAAAAAAAAAAAAAAAAAAAAAAAAA

Figure 2. The complete mRNA of tobacco protein plant cadmium resistance 8 gene and its encoding amino acids. *indicates the stop codon.

Further BLAST analysis of this protein revealed that tobacco protein plant cadmium resistance 8 shares high homology with the protein plant cadmium resistance 8 of potato(Accession number: XP_006356149, 81%), *Lycopersicon esculentum* (Accession number: XP_004241740, 80%), *Eutrema salsugineum* (Accession number: XP_006392946, 60%), *Capsella rubella* (Accession number: XP_006305669, 58%) and thale cress (Accession number: AAM62872, 57%) (Figure 3).

Based on the results of the alignment of different protein plant cadmium resistance 8 proteins, a phylogenetic tree was constructed using the ClustalW software, as shown in Figure 4. Phylogenetic analysis revealed that the tobacco protein plant cadmium resistance 8 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 protein plant cadmium resistance 8 gene was highly expressed in root, moderately expressed in stem, and hardly expressed in flower and leaf (Figure 5).

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Capsella rubella              MGRVT-EHEENPNNGTPVQQRGTPNQQTGVPASQFAPPNYQQANVNLSIG
Thale cress                   MGRVTTPSEEDSNNGLPVQQPGTPNQRTRVPVSQFAPPNYQQANVNLSVG
Eutrema salsugineum           MGRVTTPPEENPKNGFPAQQTGTP--------SQFAPPNYHQANVKLSVG
Potato                        MGRVEANNEEETSQAESGTEPAASQPQ-----QLQGVQGVYQSPPHLTIG
Lycopersicon esculentum       MGRVEANNEGETSQAESGTEPAASQPQ-----QFQGVQSVYQSPSHLTIG
Common tobacco                MGRVEENNEIETPKPGESGEPVASQPPP----QYQGVKDVQPPSPSQPIG
                              ****    * :. :     :  :.        .  .  .   .    .:*

Capsella rubella              RPWSTGLFDCHEDQANALMTTIAPCVTFGQITEVVDEGDMTCPLGTFMYL
Thale cress                   RPWSTGLFDCQADQANAVLTTIVPCVTFGQIAEVMDEGEMTCPLGTFMYL
Eutrema salsugineum           SPWRTGLFDCQEDQTNAVMTSILPCVTFGQIAEVVDEGEMTCPLGSFIYL
Potato                        APWSTGLFDCHLDQTNAVMTAFLPCVTFGQIAEVLDAGQMTCPLGTFIYM
Lycopersicon esculentum       APWSTGLFDCHLDQTNAVMTAFLPCVTFGQIAEVLDAGQMTCPLGTFIYM
Common tobacco                APWSTGLFDCHLNQINAVMTSFLPCVTFGQIAEVLDAGEMTCPLGTFIYL
                               ** ******: :* **::*:: ********:**:* *:******:*:*:

Capsella rubella              LMMPALCSQWVMGSKYREKMRRKFNLVEAPYSDCASHLLCPCCALCQEYR
Thale cress                   LMMPALCSHWVMGSKYREKMRRKFNLVEAPYSDCASHVLCPCCSLCQEYR
Eutrema salsugineum           LMMPALCSQWVMGSKYREKIRRKFNLVEAPYSDCITHVFCSCCALCQEYR
Potato                        LMMPAICSQWIMGSKYRTQLRQRYNLVEAPYSDMISHMFCPCCSLCQEFR
Lycopersicon esculentum       LMMPAVCSQWIMGSKYRTQLRQRYNLVEAPYSDMISHMFCPCCSLCQEFR
Common tobacco                LMMPAVCSQWIMGSKYRTKLRQKYNLVEAPYSDIVSHIFCPCCSLCQEFR
                              *****:**:*:****** ::*:::*********  :*::*.**:****:*

Capsella rubella              ELKIRNLDPSLGWNGILAQG--QYGSEAPTFAPTNQYMSK
Thale cress                   ELKIRNLDPSLGWNGILAQGQGQYESEAPSFAPTNQYMSK
Eutrema salsugineum           ELKARNLDPSLGWNGILAQRQGHYESEAPSSAPPNQYMSK
Potato                        ELRNRGLDPALGWNGIVAQQ--HYGNQQVNQAPQVQSMSK
Lycopersicon esculentum       ELRNRGLDPALGWNGIVAQR--HYGNQQVNQAPQVQSMSM
Common tobacco                ELQHRGLDPALGWNGIVAQQ--HYGNQQVNQAPQVQSMSK
                              **: *.***:******:**   :* .:  . **  * **
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Figure 3. The alignment of the proteins encoded by tobacco and other protein plant cadmium resistance 8 genes

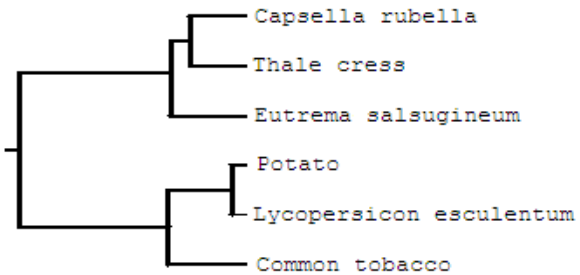

Figure4. The phylogenetic tree for six kinds of protein plant cadmium resistance 8 genes

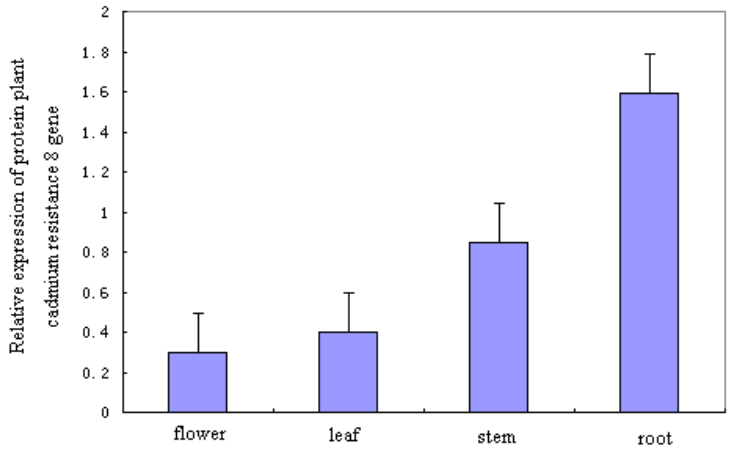

Figure 5. Expression analysis of protein plant cadmium resistance 8 gene in various tobacco tissues

DISCUSSION

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 protein plant cadmium resistance 8 genes, it can be seen that the coding sequences of protein plant cadmium resistance 8 genes were highly conserved in three solanaceae plants-tobacco, potato and *Lycopersicon esculentum*.

The phylogenetic tree analysis revealed that the tobacco protein plant cadmium resistance 8 gene has a closer genetic relationship with that of *Lycopersicon esculentum*. This implied that we can use *Lycopersicon esculentum* as model organism to study the tobacco protein plant cadmium resistance 8 gene or use tobacco as model organism to study the *Lycopersicon esculentum* protein plant cadmium resistance 8 gene.

From the tissue distribution analysis in our experiment it can be seen that protein plant cadmium resistance 8 gene was highly expressed in root. For protein plant cadmium resistance 8 can increase the plant Cd resistance (Thomine et al., 2000; Song et al., 2004; Ishikawa et al., 2012), the suitable explanation for this is that tobacco cadmium resistance is mainly existed in root.

In conclusion, we first isolated the tobacco protein plant cadmium resistance 8 gene and performed necessary sequence analysis and tissue expression profile analysis. These will establish the primary foundation of utilizing tobacco protein plant cadmium resistance 8 gene to decrease the cadmium content of tobacco and benefit the health of humans in the future.

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RESUMO: Cádmio (Cd) oriundo do fumo é um poluente que é extremamente tóxico a saúde humana. Um gene encontrado na planta de fumo que codifica uma proteína para resistência (gene 8) tem sido caracterizado para aumentar a resistência do fumo na absorção do elemento Cádmio (Cd).No experimento realizado a seqüência do mRNA de fumo que codifica uma proteína foi amplificada usando métodos de amplificação de CDNAs. A proteína completa codificada pelo gene 8 apresenta um mRNA com 887 pb com uma fita de leitura de 555 pb, a qual codifica 184 aminoácidos. A análise de BLAST demonstrou uma homologia de 81 % com o gene 8 da batateira, de 80 % com o tomateiro (*Lycopersicon esculentum),* 60 % para *Eutrema salsugineum* , 58 % para *Capsella rubella* e 57 % para o agrião. A proteína expressada pelo gene 8 para resistência do fumo a absorção do Cádmio também apresenta um forte relacionamento genético com a proteína expressa pelo gene 8 do tomateiro (*Lycopersicon esculentum).* Os perfis da expressão protéica para a proteína oriunda do gene 8 do fumo foi de grande magnitude em raiz, moderadamente expressa no caule e de difícil expressão nas flores e nas folhas. Estes resultados obtidos fundamentam o uso deste gene 8 de fumo para resistência ao Cádmio com o propósito de reduzir o teor de cádmio na planta de fumo e com reflexos benéficos para a saúde humana no futuro.

PALAVRAS-CHAVE: Fumo. Gene. Proteína 8 para resistência ao Cádmio. Padrão de expressão.

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