Molecular Cloning, Characterization and Expression Pattern of Tobacco (Nicotiana Tabacum) Acid Phosphatase Gene

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Abstract: The complete coding sequence of tobacco (nicotiana tabacum) acid phosphatase gene was amplified by RT-PCR. The open reading frame(ORF) of tobacco acid phosphatase gene was 792 bp which encodes a protein of 263 amino acids. Sequence analysis revealed that the acid phosphatase of tobacco shares high homology with the acid phosphatase of lycopersicon esculentum (79%), cacao (72%), castor bean (69%), peach (64%), capsella rubella (63%) and thale cress (62%). Phylogenetic analysis indicated that the tobacco acid phosphatase gene has a closer genetic relationship with that of lycopersicon esculentum. Tissue expression pattern was also studied and results showed that tobacco acid phosphatase gene was moderately expressed in leaf and stem, and hardly expressed in flower and root. Our experiment established the foundation for further research on this tobacco gene.
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Keywords: Tobacco; molecular cloning; acid phosphatase; gene; tissue expression

1. Introduction

The product of acid phosphatase gene belongs to the phosphotyrosine protein phosphatase family and has been identified in most plants, animals and microorganisms. It functions in hydrolyzing protein tyrosine phosphate to protein tyrosine and orthophosphate. This enzyme also hydrolyzes orthophosphoric monoesters to alcohol and orthophosphate. For human, this gene had been reported to be associated with the human coronary artery disease, inflammatory bowel disease, breast cancer and other serious diseases. For plant, this gene had been confirmed to be correlated to the resistance to ralstonia solanacearum in nicotiana benthamiana. For microorganisms, this gene had been reported to be associated with the pathogenesis of Sclerotinia sclerotiorum. Multiple alternatively spliced transcript variants encoding distinct isoforms have been identified for this gene(Poussereau et al., 2001; Willour et al., 2012; Teruel et al., 2012; Gloria-Bottini et al., 2012; Alho et al. 2013a; Alho et al., 2013b; Nakano et al., 2013).

Although acid phosphatase gene plays an important role in biological processes, until today, the tobacco acid phosphatase gene has not been reported yet. In present experiment, we will isolate the complete coding sequence of this tobacco gene, subsequently perform some necessary sequence analysis and tissue expression pattern analysis. These will establish the primary foundation of understanding this tobacco gene.

2. Material and Methods

2.1. 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, and flower in the stage of anthesis were harvested and 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 Li et al. (2008).

2.2. Isolation of the coding sequence

RT-PCR was performed to amplify the complete coding sequence of tobacco acid phosphatase 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 (1 U/1μl), and 9.8 μl sterile water. The primers for tobacco acid phosphatase gene isolation were designed based on the tobacco EST sequences (GeneBank numbers FS386023 and AM848153) which are highly homologues with the coding sequence of acid phosphatase gene of lycopersicon esculentum (Table1). The PCR program initially started with a 94°C denaturation for 4min, followed by 35cycles of 94°C/1min, 50°C (Table1)/1min, 72°C

/1min, then 72°C extensions for 10min, finally 4°C to terminate the reaction. Every PCR was repeated five times. The PCR product was then cloned into PMD18-T vector and sequenced bidirectionally with the commercial fluorometric method. At least five independent clones were sequenced.

2.3. Quantitative real time PCR (qRT-PCR) for tissue expression profile analysis

qRT-PCR for evaluating the level of mRNA for acid phosphatase gene was performed on the ABI Prism 7300 Sequence Detection Systems (Applied Biosystems, Foster City, CA, USA). PCR reactions for each sample were carried out in 25µl reaction volume containing 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. For each sample, reactions were set up in triplicate to ensure the reproducibility of the results. 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, 2008).

Table 1. qRT-PCR primers for tobacco acid phosphatase, actin genes and annealing temperature

Gene	Primer sequence	Ta/ °C	Length/bp
Acid phosphatase	Forward:5'- ATGAGAATTTTGAGAGTTG-3' Reverse: 5'- TCAAGGAATGTAGTACAT-3'	50	792
Actin	Forward:5'- CCATTCTTCGTTTGGACCTT -3' Reverse:5'- TTCTGGGCAACGGAACCT-3'	56	257

2.4. Sequence analysis

The cDNA sequence prediction was conducted using GenScan software (http://genes.mit.edu/GENSCAN.html). The protein prediction and analysis were performed using the Conserved Domain Architecture Retrieval Tool of BLAST 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).

3. Results

3.1. Isolation result for tobacco acid phosphatase gene

For tobacco acid phosphatase gene, through RT-PCR with pooled tissue cDNAs, the resulting PCR products were 792bp (Figure 1).

3.2. Sequence analysis

These cDNA nucleotide sequence analysis using the BLAST software at NCBI server (http://www.ncbi.nlm.nih.gov/BLAST) 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: KF701482).

The sequence prediction was carried out using the GenScan software and results showed that the 792-bp cDNA sequence represents one single gene which encodes 263 amino acids. 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 (http://www.expasy.org/tools/pi_tool.html). The pI of tobacco acid phosphatase is 5.40. The molecular weight of this putative protein is 30171.17. Further BLAST analysis of these proteins revealed that tobacco acid phosphatase shares high homology with the acid phosphatase of lycopersicon esculentum (Accession number: XP_004235180, 79%), cacao (Accession number: EOY22799, 72%), castor bean (Accession number: XP_002510996, 69%), peach (Accession number: EMJ20017, 64%), capsella rubella (Accession number: EOA17259, 63%) and thale cress (Accession number: NP_194245, 62%) (Figure 2). Its conserved domains were identified as Acid_phosphat_B superfamily (Figure 3).

The 3-D structural evidence of the putative conserved domain is also presented in Figure 4.

Based on the results of the alignment of different species of acid phosphatase proteins, a phylogenetic tree was constructed using the ClustalW software (http://www.ebi.ac.uk/clustalw), as shown in Figure 5. The phylogenetic tree analysis revealed that the tobacco acid phosphatase gene has a closer genetic relationship with that of lycopersicon esculentum.

3.3. Tissue expression profile

Tissue expression profile analysis was carried out and results revealed that the tobacco acid phosphatase gene was moderately expressed in expressed in leaf and stem, and hardly expressed in flower and root (Figure 6).

4. Discussions

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 lycopersicon esculentum for they are both plants of solanaceae.

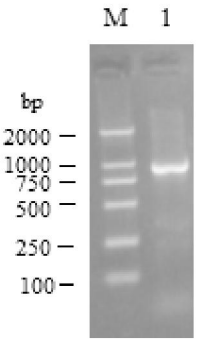

Figure 1. PCR result for tobacco acid phosphatase gene. M DL2000 DNA markers; 1, PCR product for tobacco acid phosphatase gene

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Common tobacco              -MRILRVVTFLVLFTLVTGHENLNSHMFPRPLIIEYPE--------PHHH
Lycopersicon esculentum     -MRFLGISIFLILFTFAFANEDFNTHVVDRPLIVEFFE--------NSEP
Cacao                       -MLFFKLFLLFSLLSSTFCHETFNAHLLPRPLIIQYP-----EGIETQFE
Castor bean                 MICFPRLFLFFSLFSLVFCNEQFNSHILPRPLIIEYPDNIIETETATQFK
Peach                       -MTFLKIFLFFPLLSLAFSQETFTSHLLPRPLIIEYP-----ENTETNFR
Capsella rubella            -MRILVNLLVFSLLPLAFSEENSSSYLIPRPLIFKTQLKT----------
Thale cress                 -MRILVNLILFSLIPLAFSNENSSSYLIARPLIFETQLKN----------
                             : :     .: *:. .  .*  .::::. ****.:

Common tobacco              QLKDEVQLQCTSWRFAVETNNLGPWKTIPEECGNYVRQYIEGGAYKMDID
Lycopersicon esculentum     HLKRTLHLECTSWRFAVEANNLSPWKIIPQECADYVRQYITGGAYKMEID
Cacao                       NFDEELQLQCTSWRFTVETNNLSPWKTIPEKCGGYVKDYMTGRGYTMDLE
Castor bean                 EFDNEIQLQCTSWRFAVEANNLNPWKTIPQECAGYVRDYVMGRGYQVDLE
Peach                       ELEEEFKLHCTSWRFSVEANNINPWKTIPQECAKYVKDYVTGRAYGFDLE
Capsella rubella            -IDDDVNLHCTSWRFAAETNNLAPWKTIPAECADYVKDYLMGKGYVFDVE
Thale cress                 -INDNVNLHCTSWRFAAETNNLAPWKTIPAECADYVKDYLMGEGYVVDVE
                             :.  .:*.******:.*:**: *** ** :*. **::*: * .* .:::

Common tobacco              RVSDEAGAYAKSRDLGADGKDVWVFDVDETLLSNLPYYSDHGHGLEVFDS
Lycopersicon esculentum     RVSTEAGAFAESMKLGEDGKDVWIFDVDETLLSNLPYYSQHGYGLEVFDS
Cacao                       RVANEAGVYAKSVELSGDGKDVWVFDIDETLLSNLPYYAEHGYGLEIFYP
Castor bean                 RVSNEAGVYAKSVQLSEDGKDAWVFDVDETLLSNLPYYADHGYGLEVFDP
Peach                       RVSKEAGVYAKAVELSGDGKDVWIFDIDDTLLSNLPYYADHGYGLEVFDH
Capsella rubella            RVSEEAKVYASTFESNGDGKDVWIFDIDETLLSNLPYYLEHGCGLEVFDH
Thale cress                 RVSEEAKVYASSFESNGDGKDIWIFDIDETLLSNLPYYMEHGCGLEVFDH
                            **: ** .:*.: . . **** *:**:*:********* :** ***:*

Common tobacco              VEFEKWVEKGMAPAIGSSLKLYQDVMRLGFKVFLLTGRSERHRIVTVENL
Lycopersicon esculentum     VEFDKWVEKGEAPAIGSSLKLYQDVMRLGFKVFLLTGRSERHRIVTVENL
Cacao                       VEFDKWVQRGMAPAIDPSLKLYEMVLDLGFKVFLLTGRSEEQRSVTIENL
Castor bean                 MKFDKWVEEATAPAIESSLKLYKEVRGLGFKVFLLTGRSEYQRGVTEENL
Peach                       LEFDRWVDKAMAPAIKSSLKLYEEVLGLGIKVFLLTGRSDGKRKATIENL
Capsella rubella            SKFDKWVERGIAPAIAPSLKLYQMVKEMGYKVILLTGRRENHRVVTVENL
Thale cress                 SKFDMWVEKGIAPAIAPSLKLYQKVIHLGYKVILLTGRRENHRVITVENL
                             :*: **:.. **** .*****: *  :* **:***** : :*  * ***

Common tobacco              MNAGFQDWDKLILRGSEDHGKSATIYKSEKRDEMVEEGFRLVGNSGDQWS
Lycopersicon esculentum     MNAGFQDWDKLILRGSEDHGKSATIYKSEKRNEMVEDGLRIAGNSGDQWS
Cacao                       TKAGFQSWDKLILRDSEDHGKLATVFKSEKRSKMVEEGFRILGNSGDQWS
Castor bean                 IKAGFQSWDKLILRASGDHGKLASIYKSEKRSEMVSEGYRILGNSGDQWS
Peach                       INAGFRDWHKLILRAPDEQGKLATVYKSEKRNEMEKEGYRILGNSGDQWS
Capsella rubella            RNAGFHNWDKLILRSSDDDHKTATTYKSEKRDEMVKEGYRIRGNSGDQWS
Thale cress                 RNAGFHNWDKLILRSLDDRNKTATMYKSEKREEMVKEGYRIRGNSGDQWS
                             :***:.*.*****   :  * *: :*****.:* .:* *: ********

Common tobacco              DLLGSSTSIRSFKLPNPMYYIP
Lycopersicon esculentum     DLLGSSASIRSFKLPNPMYYIP
Cacao                       DLLGSSPSSRSFKLPNPMYYIP
Castor bean                 DLLGISMSIRSFKLPNPMYYIP
Peach                       DILGTSMSIRSFKLPNPMYYIP
Capsella rubella            DLLGSAMSERSFKLPNPMYYIP
Thale cress                 DLLGSAMSERSFKLPNPMYYIP
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Figure 2. Alignment of the proteins encoded by tobacco and lycopersicon esculentum acid phosphatase gene

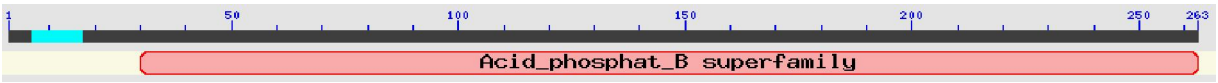

Figure 3. Acid_phosphat_B superfamily domain of the protein encoded by tobacco acid phosphatase gene

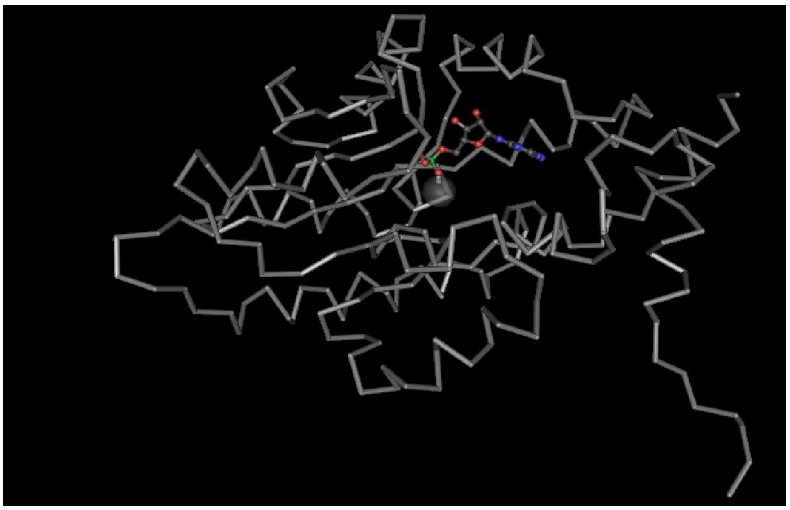

Figure 4. The 3-D structural evidence of the putative conserved domain of tobacco acid phosphatase protein

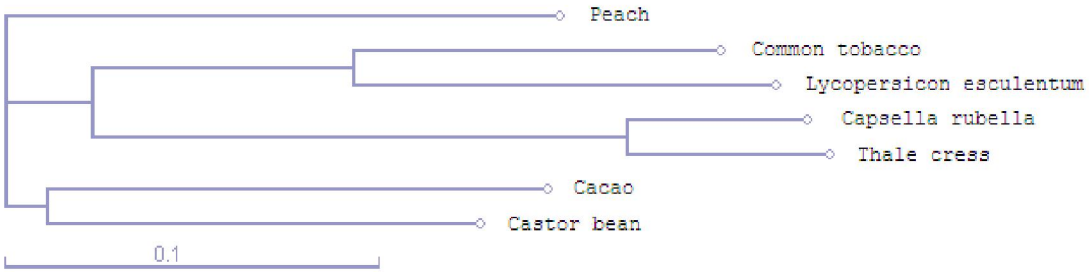

Figure 5. The phylogenetic tree for seven kinds of acid phosphatase genes

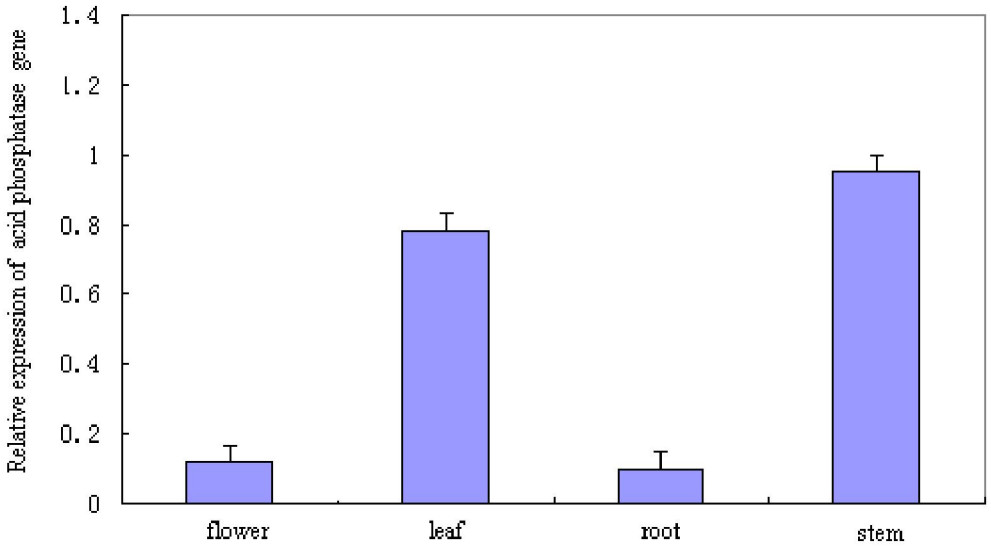

Figure 6. Expression analysis of acid phosphatase gene mRNA in various tissues

From the sequence analysis of acid phosphatase genes, it can be seen that the coding sequences of acid phosphatase genes were highly conserved in two solanaceae plants-tobacco and lycopersicon esculentum. This implied that we can use the lycopersicon esculentum as model organism to isolate some tobacco genes based on the coding sequence information of lycopersicon esculentum. Isolation of the tobacco acid phosphatase gene in this experiment further validated that is an effective method. From sequence analysis, we also noticed that the coding sequences of acid phosphatase genes from different plants do not show complete identity. This implied that these acid phosphatase genes will have some differences in functions. These merit further study.

From the tissue exression distribution analysis in our experiment it can be seen that acid phosphatase gene was obviously differentially expressed in some tissues. For acid phosphatase functions in hydrolyzing protein tyrosine phosphate to protein tyrosine and orthophosphate, and hydrolyzing orthophosphoric monoesters to alcohol and orthophosphate(Poussereau et al., 2001; Willour et al., 2012; Teruel et al., 2012; Gloria-Bottini et al., 2012; Alho et al. 2013a; Alho et al., 2013b; Nakano et al., 2013). The suitable explanation for this is that these biological activities related to the mRNA expression of this tobacco gene were presented diversely in different tissues in the stage of anthesis.

In conclusion, we first isolated the tobacco acid phosphatase gene and performed necessary sequence analysis and tissue expression profile analysis. This established the primary foundation for further research on this tobacco gene.

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