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CLONING AND EXPRESSION PATTERN ANALYSIS OF *MmPOD12* GENE IN MULBERRY UNDER ABIOTIC STRESSES

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KEYWORDS

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MmPOD12 gene

Gene cloning and expression

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ABSTRACT

A full-length cDNA denominated as *MmPOD12* for peroxidase in mulberry(*Morus alba*), an enzyme involved in the respiration, photosynthesis, and the oxidation of auxin, was cloned from ‘Yu71-1’ a variety of mulberry using a rapid amplification of terminal (RACE) approach. The full cDNA of *MmPOD12* has 1482 base pairs (bp) in length with an open reading frame (ORF) 1050 bp encoding a protein of 349 amino acids residues with a predicted molecular weight of 53.92 kDa and an isoelectric point of 9.35. Sequence analysis revealed that *MmPOD12* shares homology with the *Morus notabilis* (*M. notabilis* C.K.Schn) and has closely related to green plum, strawberry and pear. The expression patterns of *MmPOD12* treated with drought, salt and hormones stresses were examined using real-time quantitative PCR (RT-qPCR). These experiments caused significant up-regulation of the expression of *MmPOD12* under drought and salt stress. The highest expression level of *MmPOD12* appeared at 2d for salt stress, and 7d for drought stress, while a significant fluctuation of *MmPOD12* expression was detected after ABA and SA stresses. These findings provide a basis for future functional analyses of *MmPOD12* gene in Mulberry.

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1 Introduction

Peroxidase (POD) widely exists in biological world and it plays an extremely important physiological role in plants as it presents from the first hours of a plant's life until its last moments. POD is not the single-minded enzyme of hydrogen carrier except for H_2O_2 but it can also catalyze the oxidation reaction of Phenolic, Cytochrome C, Vitamin C, Nitrite, Colorless dye, Indole, Amine and Inorganic ions (especially *Iodide ion*) with H_2O_2 . Besides, due to POD can catalyze the decomposition reaction of IAA, a kind of hormone that can promote plant cell elongation, it also played a role in plant tissue differentiation, seed germination and fruit maturity (Passardi et al., 2005).

In addition, POD is very sensitive to the variety of adverse environmental conditions. POD isoenzyme will be produced quickly when plants are infected by bacteria, and rapid induction of POD isoenzyme was related to the *denovo* synthesis according to the experiments about protein synthesis inhibitors (Hiraga et al., 2001). The level of POD activity was higher when plants were treated with low concentration of ethylene or attacked by pathogens (Tognolli et al., 2002). There are many studies which demonstrated that POD can eliminate the effect of injury caused by H_2O_2 during the processes of metabolic in plants (Wu & Yu, 1994; Liang et al., 2003). Further, some studies suggested that POD activity of litchi fruit stored at room temperature was higher than the wet storage. POD have multi functional gene in *Ginkgo biloba* as it has the potential function on defense aspects, such as it participates in the removing of heavy metal pollution and dealing with the damage (Cheng et al., 2010).

Under some abiotic stress, the activity and expression level of POD were affected. There are some reports which asserted role of POD in overcoming salt stress in plants and the level of POD increased with inducing some stress (Zhu, 2002; Narayanan et al., 2005; Dai et al., 2015). Further, Dai et al. (2015) observed higher POD activities in leaves of *Asparagus bean* seedling immediately after inducing salt stress by NaCl (150 mmol/L). There's a study which found POD activity in leaves of *Populus Euphratica* and it increase with the aggravation of drought stress (Wang et al., 2013). Other papers also reported that drought stress induced the level of POD activity in *Crassulaceae* plant (Wen et al., 2014) and *Lagerstroemia indica* seedling (Liu et al., 2015). Studies on role of gene expression on POD activity suggested that under stresses of Absciscic acid (ABA) and Salicylic acid (SA), the level of POD activity change (Fang et al., 2014; Fang et al., 2014; Xu et al., 2015). Study on the effect of exogenous ABA induced stress on the seed germination and cold tolerance of winter rape seedlings, showed that the level of POD activity significant increased and the cold resistance is optimum when ABA concentration is 30 mg/L (Fang et al., 2014).

In this study, cloning of the POD12 gene in Mulberry based system on the expressed sequence tags (ESTs) from mulberry

cDNA library was constructed by following the method of Zhao (2008) and Fang et al. (2008), and we named it *MmPOD12*. The purpose of this study is to get the full-length sequence and analyzed the expression of the gene under different kinds of stresses in mulberry. These results provide a new breeding strategy for improving mulberry resistance.

2 Materials and Methods

2.1 Plant materials

Mulberry (*Morus alba*) variety 'Yu71-1', obtained from the Sericultural Research Institute, Chinese Academy of Agricultural Sciences, Zhenjiang, China, was used as the experimental material. To analysis *MmPOD12* gene expression pattern under various stresses, 'Yu71-1' grafting seedlings were grown under standard conditions that the temperature is maintained at 25°C and the photoperiod is 12 h in an incubator, until the winter shoots reached approx. 30 cm in length (50 d).

2.2 RNA isolation and synthesis of the first strand cDNA

Total RNA was isolated from fresh buds (approx. 90 mg) of the grafted Mulberry seedlings using the RNAPrep Plant kits (TaKaRa Biotechnology Co. Ltd., Dalian, P. R. China) and following the manufacturer's protocol, and then stored at -80°C re-suspended in 0.1% (v/v) diethylpyrocarbonate (DEPC)-treated water. RNA quality was determined with UV spectrophotometer and by 1.0% (w/v) agarose gel electrophoresis.

The first strand cDNA was synthesized from 9 µl total RNA (1.0 ng ml⁻¹) from the previous step using the RNase H-Reverse Transcriptase M-MLV kit (TaKaRa Biotechnology Co. Ltd.). Following the manufacturer's instructions, the reaction conditions are 42°C for 60min with 4.0 µl oligo-dT (100 µg ml⁻¹) and adaptor primer in a total volume of 20 µl.

2.3 Molecular cloning of the full-length *MmPOD12* cDNA

The first strand cDNA was used as the template for PCR in gene cloning. The forward and reverse primers were designed according to the EST with the inference function from the mulberry cDNA library (*MmPOD12* Forward primer: 5'-TAGATGCCACCGACACGGT-3'; *MmPOD12* Reverse primer: 5'-ACTTGGATTCTAGCAGAGC-3'). The RT-PCR reactions system were performed in a total volume of 50 µL contained 1.0 µl first-strand cDNA, 41 µl ddH₂O, 1 µl each gene-specific primer, 0.5 µl dNTPs (10 mM), 5 µl buffer, and 0.5 µl Taq DNA polymerase (5 U/mL) (TaKaRa Biotechnology Co. Ltd.). The PCR amplification condition: initial denaturation at 94°C for 5 min firstly; then there are 32 cycles of denaturation at 94°C for 35 s, annealing at 60°C for 45 s, and elongation at 72°C for 1 min; finally, extension at 72°C for 10 min.

The RT-PCR products were detected by gel electrophoresis (1% agarose gels) and purified following the Takara Agarose

Gel DNA Purification Kit (TaKaRa Biotechnology Co. Ltd.) following the manufacturer's protocol. The purified fragment was then cloned into the pMD18-T vector and sequenced. It was reported that cDNA sequence is the *MmPOD12* gene, but 3'-UTR and 5'-UTR is incomplete. In order to get the full length, we used 3' and 5' rapid amplification of cDNA ends (RACE) technology using the Clontech SMART™ RACE Kit (TaKaRa Biotechnology Co. Ltd.).

In 3' end reverse transcription reaction, the cDNA which was synthesized from 9 µL total RNA by Reverse Transcriptase M-MLV (RNaseH⁻) at 42°C for 1h with 4 µL 3'ap primer (in a total volume of 20µl), was used as a template. The gene-specific primer is GSP1:5'-ACAACAGCAGCATCGCCAACC-3'). 3'RACE-PCR amplifications condition: denaturation at 94 °C for 5 min; followed by 35 cycles of denaturation at 94 °C for 40 s, annealing at 62°C for 45 s, and elongation at 72 °C for 60s; with a final extension at 72°C (Tong et al., 2013).

The 5' end reverse transcription reaction following the instructions of SMART™ RACE cDNA Amplification Kit (CLONTECH Co. Ltd.). The 5'-RACE reaction system as followed: 1µl cDNA template, 17.25µl ddH₂O, 1µl 5' end specific primer (GSP2 : 5'-ATGACAGGGTCTTGGGTGGGATAGAG-3'), 1µl common primer, 2µl dNTP, 2.5µl buffer, and 0.25µl rTaq DNA polymerase (5U/mL), in total 25µL; and the reaction condition is that: initial denaturation at 94°C for 5 min; then 5 cycles of denaturation at 94°C for 30 s, annealing at 72°C for 30 s, elongation at 72°C for 2 min; then 5 cycles of denaturation at 94°C for 30 s, annealing at 68°C for 30 s, elongation at 72°C for 2 min; and then followed by 25 cycles of denaturation at 94 °C for 30s, annealing at 66°C for 30s, elongation at 72 °C for 2 min; and the final extension at 72°C for 7 min. The 3' and 5' ends RACE products were all analyzed by Gel electrophoresis.

2.4 Bioinformatics analysis of the *MmPOD12* gene

The cloned gene sequence was analyzed for identity and homology using the National Center for Biotechnology Information (NCBI) online Search Tool (BLAST) (<http://www.ncbi.nlm.gov/>). And the ORF of *MmPOD12* was using the ORF finder program at NCBI (<http://www.ncbi.nlm.nih.gov/gorf/orf.cgi>) and downloading the homology nucleotide sequences from the database. Then use the DNA Star software to splice various DNA fragments and analyses it.

The *MmPOD12* protein structure and functional domains were predicted using the tools of ExPASy (<http://prosite.expasy.org/prosite.html/>) and PSORT the software on line. The molecular mass and the theoretical isoelectric point were predicated using the software DNASTar and ExPASy-ProtParam (<http://web.expasy.org/protparam/>). Subsequently, using the ClustalX program and DNAMAN software to align and compare the multiple amino acid

sequences of POD genes from different species. The on line software SWISS-MODEL (<http://swissmodel.expasy.org/>) was used to the prediction of *MmPOD12* protein tertiary structure. Finally, software MEGA5.1 and clustalx were used to generate the phylogenetic tree of the POD proteins from different species by the neighbor-joining (NJ) method. The bootstrap analysis based on One thousand replicates, and the Protein domains were predicted using SMART (<http://smart.embl-heidelberg.de/>) package.

2.5 *MmPOD12* expression patterns under stresses using qRT-PCR

Mulberry (*M. alba*) 'Yu71-1' grafting seedlings were planted in the same specification pots, and each pot containing only one seedling. The growing conditions were controlled with a 16h photoperiod and 25 °C/22 °C (day/night, respectively). After approximately 2 months, when the shoots had reached approx. 20 cm in length, the seedlings were subjected to salt (0.3mol/L NaCl, irrigate), drought (PEG-6000, 20%), ABA (0.1mol/L, spray) and SA (0.1mol/L, spray) respectively.

The leaf samples (approx. 1.0g) were collected in 6h, 12h, 1d, 2d, 3d, 4d, 16d after the initiation of salt treatment; in 2d, 4d, 8d, 10d, 16d after the initiation of drought treatment; and in 2h, 4h, 6h, 8h, 10h, 1d, 2d, 3d after the initiation of ABA and SA treatment respectively.

To reveal the putative biological function of the *MmPOD12* protein, qRT-PCR method was used to analysis the expression levels of *MmPOD12* gene under the four abiotic stresses. The first-strand cDNA was reversely transcribed from total RNA as the directions of the Prime script RT Reagent Kit (TaKaRa Biotechnology Co. Ltd, Dalian, P. R. China), and PCR was carried out following the SYBR Premix Ex Taq Kit (TaKaRa Biotechnology Co. Ltd.) directions. The reaction system was performed in total 20µl volume, containing 10µl SYBR® Premix Ex Taq™, 0.5µl Rox Reference Dye (50×), 0.5µl each primer solution, 1.5µl reverse transcription product, and then add RNase-free water. The mulberry Maactin gene (β-actin) (GeneBank access No. DQ785808) was used for internal control to allow for normalization by visual inspection of mRNA level. The Forward primer (β-actin-F: 5'-GACAATGGAAGTGAATGG-3') and the Reverse primer (β-actin-R: 5'-GACCTCCAATCCAGACA-3') were used for PCR amplification. The reaction conditions as follows: first, initial denaturation at 95°C for 10min; then, followed by 45 cycles of denaturation at 95°C for 15s, annealing at 58°C for 20s, extension at 72°C for 20s; and then at 95°C for 15s, at 60°C for 1min; finally, end at 95°C for 15minutes.

The result of PCR was analyzed by the Applied Biosystems 7300 System SDS Software and analyzing RT-PCR data by the comparative CT method (Livak & Schmittgen, 2001). Then calculating the average values for *MmPOD12* gene expression in the two biological replicates, and the standard errors.

Figure 1 Full-length cDNA cloning of the mulberry *MmPOD12* cDNA.

- (A) RT-PCR amplification of a cDNA fragment from the mulberry *MmPOD12* gene. Lane 1, Product of RT-PCR.
 (B) 3'-RACE amplification products of a cDNA from the mulberry *MmPOD12* gene. Lane 1, Product of 3'-RACE.
 (C) 5'-RACE amplification products of a cDNA from the mulberry *MmPOD12* gene. Lane 1, Product of 5'-RACE. Lanes M, DL2000 DNA markers (TaKaRa Biotechnology Co. Ltd.).

3 Results

3.1 Gene cloning and bioinformatics analysis of *MmPOD12*

The mulberry cDNA library screening technique and the clone technology combining bioinformatics analytical methods were used to obtain a cDNA of the *Mulberry POD* gene and the RT-PCR products were analyzed by gel electrophoresis (1% agarose) (Gao et al., 2010) and get a expected size (approximately 500 bp) band was determined according to the EST sequence (Figure. 1A) (Fang et al., 2008). 3' and 5' RACE technology was used to obtain a full-length cDNA of *Mulberry POD* gene, which contained a 780 bp fragment (3'-RACE product, Figure. 1B) and a 750 bp fragment (3'-RACE product, Figure. 1C), then the full-length cDNA of the mulberry *POD* gene was obtained. This is the first time cloned *POD* gene of mulberry, and it was designated the gene as *MmPOD12* (Patent number:101510163922.9). Sequence analysis showed that the isolated cDNA from 'Yu71-1' is a small gene with a 1482 bp full-length, which contains a 1050 bp ORF encoding a protein of 349 amino acids, and it was predicted that the molecular weight of the protein is 53.92 kDa and the isoelectric point is 9.35 (Figure. 2).

According to software Batch CD-search (<http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>), we find that there is a conserved domain contained by *MmPOD12* gene structure. This conserved domain can be regarded as position-specific score matrices (PSSMs) when identifying the conserved domains in protein sequences by RPS-BLAST.

3.2 Sequence analysis of *MmPOD12*

The homologous alignment and deduced amino acid sequences of *MmPOD12* were analyzed using the software Clustal X2.0 program (<http://www.clustal.org/>) and Vector NTI Advance 11. The results showed that the length and structure of *MmPOD12* homologous sequences are relatively similar with other seven species. Homology analysis revealed that *MmPOD12* is highly conserved among mulberry different species, but there are three different amino acids in 'Yu71-1' and *Morus notabilis*, this means there are particularities in different species.

According to the *MmPOD12* amino acid sequence structuring the evolutionary tree by MEGA5.1 Software. The results were visualized in Figure. 3 that the length and structure of the 16 species amino acid sequences are very conservative. There is relatively close homology between 'Yu71-1' and *Morus notabilis*, but the homology is far between 'Yu71-1' and *Eucalyptus grandis*, *Grape*, *Populus euphratica*, *Populus trichocarpa* and *Litchi*.

3.3 Stress-induced *MmPOD12* gene expression

To further analyze whether the level of *MmPOD12* gene expression is induced by various abiotic stresses, it was monitored that the *MmPOD12* mRNA levels under abiotic stresses by qRT-PCR (Figure. 4) illustrates that, the expression level of *MmPOD12* gene fluctuates obviously under salt stress, the overall trend is decreasing at the initial of salt treatment, followed there is a sharpest rise and up to a maximum, and then decrease slowly (Figure. 4A).

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1
AGCGAAAGCTTTGTATATAATAAATGATTTACCCACCATAGTGCATAAAAAAGTGCTGCTGATGAAGTCTCTTGGTCTAATAT
CACTGCTCTTGACGCGCATGATTCTGTTTTCTGCTTCGTGGTGGTGACTATGCATCACGATCACCTGAA 152
153 atgagctacagataacgtgtgtgtgatgacgatgatgatgatctgtgaagtgtgtcgtttatgcacataaatcatcagatgtg
M A T D N V V V V M T M M M I C V S C V V Y A H K S S D V
243 cctctgtcgaaggactgtcttgagcttctatgacaagagctgtccagatgctgagtcacataagaactcaactcaagaaggtttc
P L A E G L S W S F Y D K S C P D A E S I I R T Q L K K V F
333 aacgaggacattggccaagctgctgcttctcgtccattccatgactgcttgttcagggtgtgacgggtcagtggttggac
N E D I G Q A A G L L R L H F H D C F V Q G C D G S V L L D
423 gggtcggcagtggtggcggacgagaagaacgcctccgaacctgagtttaagagcgaaggctttgagataataaacgatctacgcgc
G S A S G P D E K N A P P N L S L R A K A F E I I N D L R A
513 agagtcataagaagtgcggtcagtcgtcttctgttcgatacgtctgattgcagcagtgattctgttctgtcagtggtgcct
R V H K K C G R V V S C S D I A A I A A R D S V F L S G G P
603 gactatgcagtagcattggaagaagacgggtgacatttgcacaagagcagtaactatagccaacctctcatcaacaagaac
D Y A V P L G R R D G L T F A T R A V T I A N L P S S T S N
693 gctagcactatgctgccttcttgcacgaaaaacatagatgc actgacgcggttgccttccagcggccacaccattggccttagc
A S T I V A F L A T K N L D A T D A V A L S G G H T I G L S
783 aattgctcctctttaacaacgcctctatcccaacacacccctgcatggaccagacttttgcaaaaacctaaggcgatttgcctc
N C S S F N N R L Y P T Q D P V M D Q T F A K N L K A I C P
873 acgaacatcaggttgcactactgtgctcgatattcgaagccataatgccttggacaacaagtactatgtcaaccttgaaccgcaa
T N I T V A T T V L D I R S P N A F D N K Y Y V N L M N R Q
963 ggactcttcacatctgatcaggattgtacagctacagtaagaccaaggacattgcatcaactcgcggttaataatcactctcttc
G L F T S D Q D L Y S Y S K T K D I V I N F A V N Q S L F F
1053 gaataatcatcatcgccatgactaaaatggccaattaagtgtcttgacagcgaccaaggtgagatagtgccaattgctctgctagg
E K F I I A M T K M G Q L S V L T G S Q G E I R A N C S A R
1143aatccaagtaaggataactggcgtctctatagatgtgacgaactgtcggcgttctaa
N P S K D N L A S L Y E Y V D N L S A F *
GGGCTTTAGGTTTTGGCTTTGTTTCAAAGATGTTCTTTTGTAAAAAATAAAAAAAAAAAAAAAAAAAAAAAAAAAAAACC
TTGTGGGGCCTCTC
CGTGGGGTAATGCAGACCATCCTTTTGGGGGGTTCGGCTTTGTCCACCTTGTCCCAAGTTTGTGATTAATTGTTTATGGGT
TGAATGGTAAGG
TCTTTGACCATCCATCGCGAGACAAAAAACTTGGGGGTGTTGGTCTTCTTTTGTAGTAAATATAAGGGGGCAATCTAGTGT
TAAGT 1482

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Figure 2 The nucleotide and deduced amino acids sequence of mulberry *MmPOD12*
ATG indicates the start codon ; TAA indicates the stop codon

After the drought stress, the expression level of *MmPOD12* gene change little at the initial days, it begin to increase in 8d and continued to grow (Figure. 4B). The expression level of *MmPOD12* under ABA and SA stresses

showed a significant wave as it increase firstly and then decrease (Figure.4C and Figure.4D). According to the qPCR analysis, we initially speculated that *MmPOD12* is related to mulberry resistance, especially in salt and drought stresses.

Figure 3 The phylogenetic tree based on amino acid sequence of *MmPOD12* and other homologues sequences.

Figure 4 Analysis of *MmPOD12* expressing under drought, salt, ABA and SA stresses.

(A): Analysis of *MmPOD12* expressing in 0h, 6h, 12h, 1d, 2d, 4d and 16d under salt stress. (B) Analysis of *MmPOD12* expressing in 0d, 0.5d, 2d, 4d, 8d, 10d and 16d under drought stress. (C) Analysis of *MmPOD12* expressing in 0d, 2h, 4h, 6h, 8h, 10h, 1d, 2d and 3d under ABA stress. (D) Analysis of *MmPOD12* expressing in 0d, 2h, 4h, 6h, 8h, 10h, 1d, 2d and 3d under SA stress.

4 Discussions

In this study, a full-length cDNA sequence of *POD* from mulberry variety 'Yu71-1' was obtained for the first time by cloning technology. It was analyzed the gene's sequence, expression pattern and predicted the protein coded by it and explored the expression level of *MmPOD12* under various abiotic stresses. In this study, it was reported that *MmPOD12* can compared with the *POD* in other species with a higher homology, an identical conserved regions and a similar tertiary structure. When mulberry plants were in adverse environments such as salt and drought, the expression level of *MmPOD12* will adjust to response to environmental stresses.

The adjustment mechanism of *MmPOD12* was understood preliminarily. The results of qRT-PCR analysis shows that the expression level of *MmPOD12* mRNA significantly increased under salt stress, and the expression level up to the highest in 6 h after treatment. This indicates that the adversity like salt affect *MmPOD12* gene expression and over expression. This present result was consistent with the previous reports that the *POD* genes played an important role during the plants' physiological mechanisms adapting to salinity change (Du et al., 2011).

Under drought stress, the transcriptional level of *MmPOD12* mRNA will increase gradually with the extension of time and there is a sharp increase peak at the later period. This indicates that, under mild and moderate drought treatment, *MmPOD12* activity was modest overall increase as the plants against the damage caused by drought stress through other path mainly; and when mulberry plants under severe drought environment, *MmPOD12* activity increased significantly as it participating in resisting the drought stress. This is consistent with other species with strong drought resistance (Peng et al., 2005).

Under ABA and SA stresses, the expression levels of *MmPOD12* increased, and the effect of SA more obvious than ABA. This suggests that spraying exogenous ABA can improve *POD* activity and this is consistent with the study result of Fang et al. (2014).

To summarize, a full length cDNA of *MmPOD12* was isolated from mulberry variety 'Yu71-1' and characterized in this paper. Multiple alignments and bioinformatics analysis results showed that the deduced *MmPOD12* had high similarity to other plant *PODs*. Expression profiles of *MmPOD12* under different treatments suggest that *MmPOD12* was a stress-responsive gene, especially to salt and drought. The cloning,

characterization and expression analysis of *MmPOD12* will be helpful to understand more about its role in the resistance to stresses for plant, which provide the basis for improving the ability to anti-stress by genetic manipulation in the near future.

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Conflict of interest

Authors would hereby like to declare that there is no conflict of interests that could possibly arise.

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