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

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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 H₂O₂ 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 H₂O₂. 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 papers which demonstrated that POD can eliminate the effect of injury caused by H₂O₂ 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 important 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 mM). 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 Abscisic 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 got 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 RNase H–Reverse Transcriptase M–MLV kit (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 μl⁻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 60 min with 4.0 μl oligo-dT (100 μg μl⁻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'-TAGATGCCCACCGACGCT-3'; MmPOD12 Reverse primer: 5'-ACTTGGATTCAGCAGACG-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 (10mM),5 μl buffer, and 0.5 μlTaq 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 45s,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
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 1 h with 4 µL 3’ap primer (in a total volume of 20(µl), was used as a template. The gene-specific primer is GSP1: 5’-ACAAACGCGATCGCAACC-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 (GSP22: 5’-ATGACAGGGTTCTGGGTGGGATAGAG-3’), 1µl common primer, 2µl dNTP, 2.5µl buffer, and 0.25µl rTaq DNA polymerase (5U/µl), in total 25µl; 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 30 s, 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/orf/orf1.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 predicted 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 MEGAS.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 (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/LNaCl, 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 TaqTM,0.5µl Rox Reference Dye (50x),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’-GACAATGGAACTGGAATGG-3’) and the Reverse primer (β-actin-R: 5’-GACCTCTCAATCCAGACA-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,at60°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.
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 (5’-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 regard 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 mean there are particularities in different species.

According to the MmPOD 12 amino acid sequence structuring the evolutionary tree by MEGA5.1 Software. The results was visualized in Figure.3 that the length and structure of the16 species amino acid sequences are very conservative. There is relatively close homology between ‘Yu71-1’ and Eucalyptus grandis, Grape, Populus euphratica, Populus trichocarpa and Litchi.

3.3 Stress-induced MmPOD12 gene expression

To further analysis 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).
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.
4 Discussion

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 MnPOD12 under various abiotic stresses. In this study, it was reported that MnPOD12 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 MnPOD12 will adjust to response to environmental stresses.

The adjustment mechanism of MnPOD12 was understood preliminarily. The results of qRT-PCR analysis shows that the expression level of MnPOD12 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 MnPOD12 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 MnPOD12 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, MnPOD12 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, MnPOD12 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 MnPOD12 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 MnPOD12 was isolated from mulberry variety ‘Yu71-1’ and characterized in this paper. Multiple alignments and bioinformatics analysis results showed that the deduced MnPOD12 had high similarity to other plant PODs. Expression profiles of MnPOD12 under different treatments suggest that MnPOD12 was a stress-responsive gene, especially to salt and drought. The cloning,
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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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