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CLONING, SEQUENCE ANALYSIS AND INDUCED EXPRESSION STUDIES OF A CHITINASE GENE M-CHITINASE FROM MULBERRY (MORUS L.)

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Abstract: A full-length cDNA sequence coding for Chitinase in mulberry, which we designated M-chitinase (GenBank accession number: HQ117891) was cloned based on mulberry expressed sequence tags (ESTs) isolated from the cDNA library. Its full open reading frame was obtained by RACE and RT-PCR. Sequence analysis showed that the M-chitinase gene is 1392 bp in length and contains a 60 bp 5'-UTR (un translated region) and a 255 bp 3'-UTR. Its open reading frame (ORF) is of 1077 bp long, encoding 358 amino acids with a predicted molecular weight of 38.52KDa and an isoelectric point of 4.466. Homology analysis revealed that M-chitinase gene in mulberry is highly conservative with other species including *N. khasiana*, *Zea mays* and *Zea Diploperennis*. Phylogenetic analysis based on M-chitinase gene with other 19 species revealed that mulberry shows closer relationship with *Nicotiana gossei*, *Nicotiana tabacum*, *Capsicum annuum* and *Arabis blepharophylla*. The results of semi quantitative RT-PCR analysis showed that the mRNA transcriptional level of M-chitinase in the young leaf was changed significantly under the conditions of signal transduction mechanism underlying the stress response in mulberry.

Keywords: Chitinase, cDNA library, Mulberry

INTRODUCTION

Plant chitinase is plants' metabolites produced under environmental stress conditions. It mostly exists in stems, leaves, seeds and calli of plants and mainly used for increasing the resilience of plant defense, against invasion by fungi and insect pests.

Studies have shown that it plays an important role in plant developmental regulation (Kragh *et al*., 2002), antifungal (Gao, 1999; Yu *et al*.,2000), bacteria-resistant (Zhao & Chye, 1999 ; Xu *et al*., 1996), pest-resistant (Wang & Peng, 2005), Frost (Hon *et al*., 1995), symbiotic nitrogen (Minic *et al*., 1998) and associated with human diseases (Ling & Recklies, 2004; Zhu *et al*., 2004). Chitinase has many other physiological functions, for example Chitinase is broad participates in the photosynthesis of plants and other processes (Stangarlin *et al*., 2000).

Up to now, Chitinase gene is mainly used in three aspects (Kragh et al., 1996 ; Ma et al., 2004) in the study of plants' disease resistance gene engineering: first is transforming wild bio-control strain to get new disease-resistant mechanism, second is transforming chitinase genes of the original plant, import Enhancer or changing strong promoter to increase expression of chitinase gene, third is bringing other sources of chitinase gene into the host plant to improve the expression levels of host plants.

The earlier studies of plant chitinase gene were in kidney bean. Broglie (1986) constructed the cDNA library of kidney bean using its mRNA as template, and got the chitinase gene through molecular hybridization. Shinshi et al. (1987) constructed the cDNA library of *Nicotiana tobacum* and using the stylet tagged ^{32}p to get the masccline clone. After that, Zhu et al. (1986) isolated the full nucleotide sequence of *RCH10 chitinase* gene from Rice genomic library tagged with the chitinase gene fragment of broad bean.

Mulberry (*Morus L.*), a perennial tree or shrub, is an important economic plant. It not only used for sericulture as the sole food plant for the domesticated silkworm (*Bombyx mori.*) but also for a variety of other purposes such as the production of edible fruits or useful timber (Rai *et al*., 2009). The growth and productivity of mulberry are adversely affected by abiotic and biotic stress (Pan & Lou, 2008). Only a few studies have investigated the role of chitinase gene in plants, and the exact function of Chitinase genes and encoded proteins in the stress response is still not fully understood in mulberry. Therefore, efforts to investigate the molecular adaptation mechanisms to stress and to strengthen stress tolerance are of fundamental importance of mulberry production.

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In this study, we cloned the *M-chitinase* gene based on the expressed sequence tags (ESTs) from mulberry cDNA library constructed previously (Fang *et al*., 2008; Zhao, 2008), and got the full-length sequence by RT-PCR (Gao & Zhao, 2010; Zhang *et al*., 2011) and RACE method (Guo *et al.*, 2011). The mRNA expression levels of *M-chitinase* at different developmental stages in mulberry leaves, flowers and tissues were investigated. Furthermore, changes in the transcription level of M-chitinase under SA, ABA, and NaCl stress were detected by semi-quantitative RT-PCR. In addition, Prokaryotic expression vector for expression in *E. coli* was also constructed. The purpose of this study is to lay a good foundation for understanding the signal transduction mechanism underlying the stress response and design new breeding strategies for improving mulberry production through the study of transgenic of resistance in the future.

MATERIALS AND METHODS

Plant materials and reagents: To analyze the gene expression under different conditions, mulberry variety Yu71-1 (*Morus multicaulis*) was grown under standard conditions in the National Mulberry Gene Bank of the Sericultural Research Institute, Chinese Academy of Agricultural Sciences, Zhenjiang, Jiangsu Province, China was utilized. The mulberry shoots were grafted, and then were transferred into an incubator, and maintained at 25°C and 12-h photoperiod to induce burgeoning until the winter buds grew to about 30cm in length (50d).

For the study and analysis of the expression level of M-chitinase gene at different developmental stages and in different tissues, samples were collected from a 10-year -old mulberry tree along with the same variety of Yu71-1 grown in the same field.

RNAiso Plus, Reverse Transcriptase M-MLV（RNase H^-, RNase Inhibitor, dNTP, rTaq polymerase,T4 DNA ligase, Agarose Gel DNA Purification Kit, pMD18-T Vectors were purchased from Takara (TAKARA Bio Co., Ltd.). IPTG, X-gal and all the PCR primers were synthesized by Sangon biotech (Shanghai) Co., Ltd. The host bacteria *E.coli* TOP10 was kept in Mulberry Genetic Laboratory and all chemicals used were analytical grade reagents. cDNA library screening and determination of mulberry leaves was complete by Shanghai Gene Science and Technology Research Institute (Shanghai United Gene Holdings. Ltd.).

Forward and reverse primers of RT-PCR were designed according to the EST library of mulberry leaves (Zhao, 2008).

***M-chitinase*-F: 5'- TTTGTTAGGGAAGTAGGCACC-3'**
***M-chitinase*-R: 5'- ACCACACTACAAGCAGTTGAGGCGA-3'**

RNA isolation and synthesis of the first strand cDNA: Total RNA was isolated from browses (net weight about 100 mg) of grafted mulberry seedlings using RNAiso Plus reagent. The quality of total RNA was determined using ultraviolet spectrophotometer (UVS) combined with electrophoresis. RNA sample of 1μL was diluted by 99μL DEPC water, and the absorbance (A) was measured by ultraviolet spectrophotometer (UVS). The ratio of A260/A280 was used to express the RNA purity, and then 1% extraction quality of agarose gel electrophoresis to check its quality.

The first strand cDNA was synthesized from total RNA by Reverse Transcriptase M-MLV（RNase H^-）at 42°C for 60 min with oligo (dT) adaptor primer following the manufacturer's protocol. The first-strand cDNA was used as the template for PCR in gene cloning.

Cloning of Chitinase gene in mulberry: RT-PCR reactions were performed in a total volume of 50μL including 1μL first-strand cDNA, 41μL ddH_2O, 1μL each of the gene-specific primers, 0.5μL dNTP, 5μL buffer, and 0.5μL rTaq DNA polymerase (5U/μL). The RT-PCR amplifications were performed using the following parameters: Force-denatured at 94°C for 5 min followed by 30 amplification cycles (94°C for 40s, 58°C for 40s, 72°C for 1 min), with a final extension step of 7 min at 72°C.The RT-PCR products were analyzed in 1% agarose gels and purified using Takara Agarose Gel DNA Purification Kit following the manufacturer's protocol. The purified fragment, which was confirmed to have the predicted length, was then cloned into pMD18-T vector, transforming product into host bacteria strains of *E. coli* TOP10 competent cells, coating on the LB plate containing X-gal, IPTG and Ampicillin, then pick the white spot for sequencing after cultivation, last compared the result with the original EST sequence.

Obtainment of the full-length cDNA sequence: The homology comparison showed that cDNA sequence of the *M-chitinase* gene fragment obtained only had the complete 3'-UTR and incomplete 5'-UTR. Nested reverse primers were designed according to the two sequences and 5' rapid amplification of cDNA ends (RACE) amplification was performed.

RACE-1: 5'- CCAGATGGGGTGCCTACTTCCCTAACAA-3'

Total RNA from browses was used as template to generate first strand total cDNA using Clontech SMART™ RACE Kit (Clontech Laboratories Inc., USA) in terms of manual instruction. Kit universal primers F1 is the primers for PCR amplification, forward and reverse primers is F1 and RACE-1.Amplification conditions were as follows: Force-denatured at 94°C for 5 min, followed

by 30 cycles of amplification (94℃ for 40s, 50℃ for 40s, 72℃ for 1 min) and by 72℃ for 10 min. Recycling amplification products and cloned into pMD18-T vector, then transforming ligation products into host bacteria strains of *E.coli* TOP10 competent cells. The cloning was operated by Shanghai Sangong Biological Engineering Technology & Services Co. Ltd.

Bioinformatics analysis of *M-chitinase* gene: Full length *M-chitinase* gene sequence has analyzed on NCBI Web site (http://www.ncbi.nlm.gov/). According to the amino acid sequence encoded by the gene, we selected part of homologous amino acid sequences from the NCBI for homology analysis using Clustal X software.Then carried out the phylogenetic analysis using MEGA4.1 software. At last the structural domains of protein were carried out using SMART (http://smart.emblheidelberg.de/) and Predict Protein analysis software online.

Semi-quantitative expression analysis of *M-chitinase* gene in different parts of Mulberry: Semi-quantitative RT-PCR was performed to investigate the transcriptional level of M-chitinase mRNA content in different parts of mulberry. Total RNAs were isolated from Mulberry young leaf, top bud, mature leaf, phloem, xylem, full bloom, wilting flower, mature fruit and root, respectively when test shoots of Mulberry sapling to about 20cm long. Semi-quantitative RT-PCR was carried out with 1ul cDNA as template, and mulberry *Maactin* gene (β-actin) (GenBank accession No. DQ785808) a house-keeping gene, was used as an internal control to allow for normalization by visual inspection of mRNA levels.

Induced expression analysis of *M-chitinase* gene: Salicylic acid, Abscisic acid and salt stress was carried out respectively when test shoots of Mulberry sapling to about 20cm long. Qualitative analysis was operated on LabImage 2.7.1 gel analysis software (Kapelan GmbH Co., Germany) to detect the expression level of *M-chitinase* gene under various stress condition.

Salt stress treatment:

Salt stress -induced with concentration of 300mM NaCl solution. Immersing the grafted seedling roots in 300mM NaCl solution for 12 days directly, keep temperature at 25℃, 12 hours light and 12 hours dark. Taking antithetical leaves (1-3 position leaf) of mulberry at 0 hour and the salt-induced leaves at 6 hour, 12 hour, 24 hour, 48 hour and 72 hour as experimental materials.

Salicylic acid treatment:

Aspersing the plant with 1.5mmol/L salicylic acid liquid when test shoots of Mulberry sapling to about 20cm long, continuing cultivation in greenhouse. Taking antithetical leaves and the salicylic acid treatment leaves(1-3 position leaf) of mulberry at 0 hour and the salicylic acid treatment leaves at 6 hour, 12 hour, 24 hour, 48 hour and 72 hour as experimental materials.

Abscisic acid treatment:

Aspersing the plant with 1.5mmol/L Abscisic acid liquid when test shoots of Mulberry sapling to about 20cm long, continuing cultivation in greenhouse. Taking antithetical leaves and the Abscisic acid treatment leaves(1-3 position leaf) of mulberry at 0 hour and the Abscisic acid treatment leaves at 6 hour, 12 hour, 24 hour,48 hour and 72 hour as experimental materials.

RESULTS

Cloning of Chitinase gene in mulberry: RT-PCR products were detected by 1% agarose gel, and obtained the expected size (Fig. 1). The result showed that it was basically the same sequence with related EST.

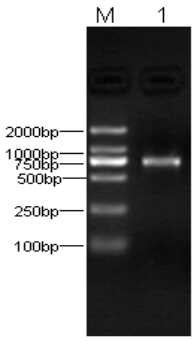

Fig. 1: RT-PCR result of mulberry gene M-chitinase
M.DL2000 DNA molecular marker; 1.Product of RT-PCR

Full-length cDNA Cloning of Chitinase gene in mulberry: To clone the full-length cDNA of the mulberry M-chitinase gene, a cloning strategy combining bioinformatics analysis and the mulberry cDNA library screening technique was used. Primers were designed according to the EST fragment of the gene encoding Chitinase. 5'RACE were performed and get a length of 700 base pairs (bp) fragment. (Fig.2) Then Entering the gene in Genbank (NO.HQ117891).It's the first time full-length cloning of *M-chitinase* gene in mulberry.

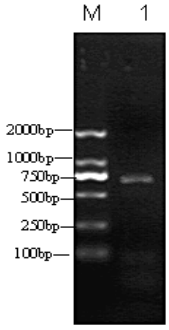

Fig.2: The amplification result of 5' RACE
M. marker DL-2000; 1.PCR amplification products

The full length cDNA sequence analysis of M-chitinase gene showed that full length of M-chitinase is 1392bp and contains a 60bp 5'-UTR (untranslated region) and a 255bp 3'-UTR. Its opening frame (ORF) is of 1077bp, encoding 358 amino acids with a predicted molecular weight of 38.52KDa33, an isoelectric point of 4.466. SMART analysis showed that the signal peptide was 25 amino-acid residue in N end (pane in Fig. 3), followed by ChtBD1 district and Pfam: Glyco_hydro_19 district.

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1    GACTCATGAACCACCTTACTACTCAGTGCAAGAGAAAAAATAAAAATAATAATAATCAAG
61                ATGATGATCAACTTCCGGAGCGCTAGTCTTATAATAATCTTGTCCCTCGGCTTGCTGCTAGGAACATCCGTGGCT
1    M M I N F R S A S L I I I L S L G L L L G T S V A
136  GAACAATGTGGAGCACAAGCTCCGGGTTCTGTGTGTCCTGATGGGTATTGTTGTAGCAACTACGGATGGTGCGGC
26   E Q C G A Q A P G S V C P D G Y C C S N Y G W C G
211  ACCTCCGACGCGTACTGTGCCCCCGGAAACTGCCAAAGCCAATGCCCCCCTCCTACTCCTCCACCACCACCGTCT
51   T S D A Y C A P G N C Q S Q C P P P T P P P P P S
286  CCACCGCCTCCACCGCCTTCACCACCTCCTCCCTATGTCCCGCCGCCTCCCCCTTCTCCCGACGACATCACCAAT
76   P P P P P P S P P P P Y V P P P P P S P D D I T N
361  ATCGTTTCTGAGTCTCTCTTCAATGAAATGCTTCTCCATCGAAACGATGATCCGAATTGTGCTGTAGGCTTCTAC
101  I V S E S L F N E M L L H R N D D P N C A V G F Y
436  ACTTACGACGGTTTCGTTACCGCCGCCGCCAGACGTTACCCTGAGTTTGGTGCTACCGGCACTCTTGAAGATCGTAAG
126  T Y D G F V T A A R R Y P E F G A T G T L E D R K
511  AGAGAGGTCGCGGCTTTCTTCGGCCAAACTTCTCATGAAACCACAGGAGGATGGCCCACAGCACCGGACGGTCCA
151  R E V A A F F G Q T S H E T T G G W P T A P D G P
586  TATACCTGGGGATATTGCTTTGTTAGGGAAGTAGGCACCCCATCTGGCTATTGTGTTCCTAGCAATGAATGGCCC
176  Y T W G Y C F V R E V G T P S G Y C V P S N E W P
661  TGTGTTCCTGGTCAAAGTTATTACGGCCGAGGACCCATTCAACTAAGCTACAACTTCAATTACGGGCAAGCAGGA
201  C V P G Q S Y Y G R G P I Q L S Y N F N Y G Q A G
736  GCCGCACTTGGATTACACCTACTTAGCAATCCCGATCTGGTTGCCACAGACGTCGTTGTATCATTCGAGACAGCC
226  A A L G L H L L S N P D L V A T D V V V S F E T A
811  TTATGGTTTTGGATGACTCCACAGGCCCCCAAACCTTCATGCCATGATGTTATAACCGGTCAATGGACGCCAACT
251  L W F W M T P Q A P K P S C H D V I T G Q W T P T
886  CCAGCAGACAGAGCAGCCGGCCGACTTCCTGGCTACGGTATGACCACCAACATCATCAATGGAGGTCTAGAATGC
276  P A D R A A G R L P G Y G M T T N I I N G G L E C
961  GGCGGCCAGGGTCCTGATGATAGGGTGGAAAATAGAATTGGCTTCTATTTGAGGTATTGTGGCTTGCTGGGAGTG
301  G G Q G P D D R V E N R I G F Y L R Y C G L L G V
1036 GACCCTGGGGATAACTTGAATTGCTATAATCAAAGTCCTTATGGGGTTACTCTTGCGAATCCAAAATATGAGATG
326  D P G D N L N C Y N Q S P Y G V T L A N P K Y E M
1111 CTCAAAATGCCTGTCTATCAGGCATAA
351  L K M P V Y Q A *
1138 TTTAATATATAATATGACGAGATCGAATAAATCATCATATGTTATGAGCTTTCTGATCATGATATGATCAAGAGA
1213 GAGCTAGTAACGTGCTACTACTATATATATATGTTGCGTGTATGTAATAAAATAAGCAGGCCTCGCCTCAACTGC
1288 TTGTAGTGTGGTGTGCTTAATTCGATCTTATGATTTATATATGTTAGTAAGATGTTTCTTTTTTTTTCCCAATT
1363 CAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
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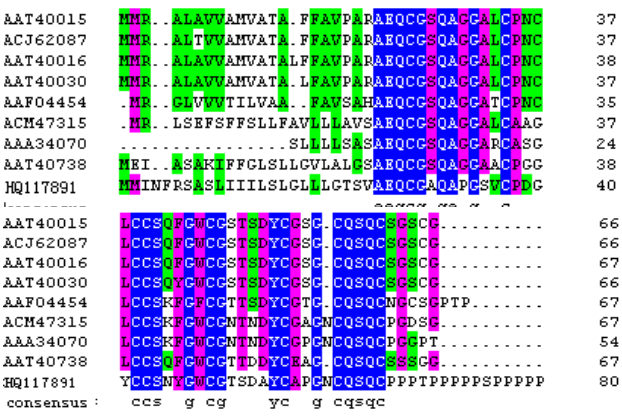
Fig.3: The full length cDNA sequence and deduced amino acids of mulberry gene *M-chitinase*
ATG indicates the start codon; TAG indicates the stop codon

Homologous alignment and Phylogenetic analysis of the *M-chitinase* gene: To understand the function and evolutionary relationship of the deduced amino acid sequence encoded by mulberry M-chitinase gene, multiple sequence alignment was carried out by Clustal X program using the amino acid sequences of M-chitinase gene and other nine species (Fig. 4).



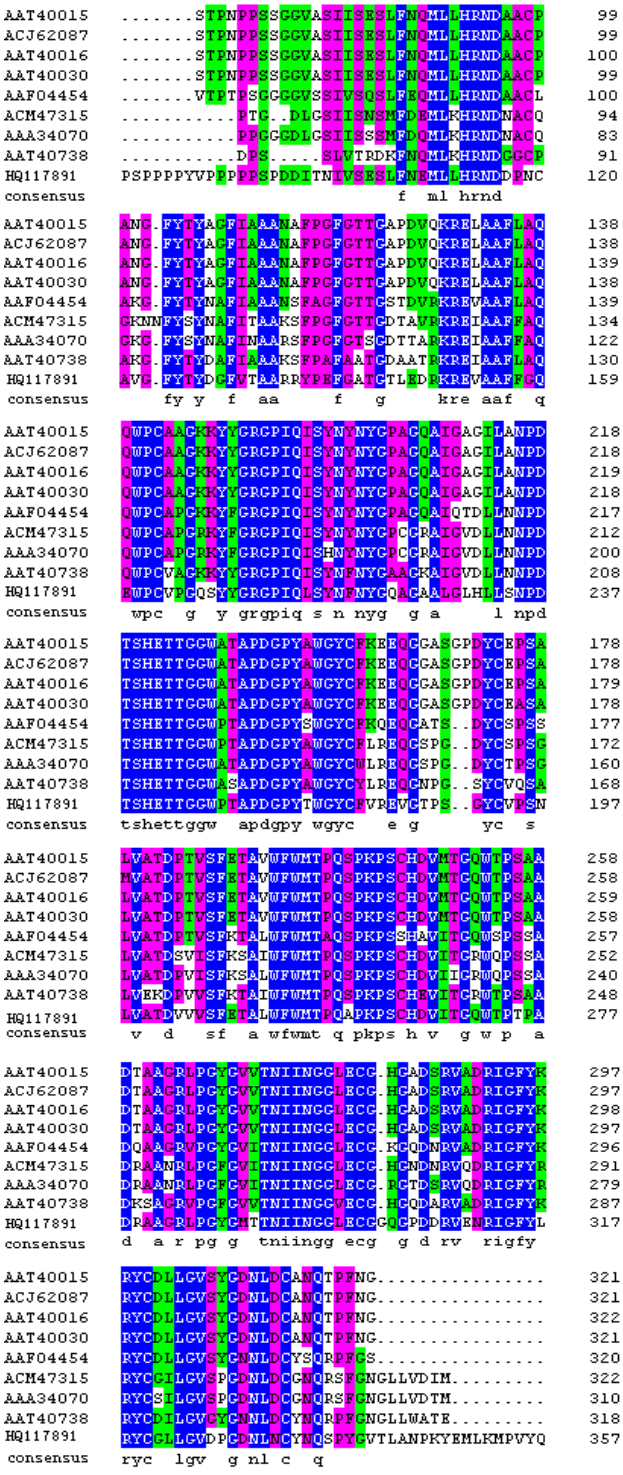


Fig.4: Multiple sequence alignment of amino acids encoded by mulberry gene *M-chitinase*

Sequence alignment indicated that all the proteins have conserved domains. Of the total mulberry M-chitinase has a highest identity with *Nepenthes khasiana* (AAT40738). Additionally, the M-chitinase sequence shared very high identity of 93% with *Zea mays subsp. Parviglumis* (AAT40015, AAT40016, and ACJ62087) and *Zea Diploperennis* (AAT40030), 92% with *Poa pratensis* (AAF04454), 91% with *Nicotiana tabacum* (AAA34070) and 89% with and *Capsicum annuum*

(ACM47315). It revealed mulberry M-chitinase has very high conservatism with other nine species.

In order to determine the evolutionary relationship between M-chitinase and other proteins, phylogenetic analysis was further carried out by MEGA4.1 program with default parameters using the amino acid sequences of M-chitinase gene from mulberry and other 19 species. The phylogenetic relationship among 20 M-chitinase gene from different species (Fig. 5) shows that the mulberry (Morus multicaulis), Nicotiana sylvestris, *Nicotiana tabacum*, *Capsicum annuum*, *Arabis blepharophylla* had a relatively high closer relationship.

Expression levels of *M-chitinase* gene at different developmental stages of mulberry leaves, flowers and tissues: To elucidate the mechanisms underlying M-chitinase gene expression in mulberry, the expression of M-chitinase was further analyzed in flowers and leaves of mulberry at different developmental stages using semi-quantitative RT-PCR. We chose mulberry β-actin gene as internal reference, and it showed that M-chitinase has a high expression in top bud, young leaf and full bloom, nevertheless, with low expressi other position (Fig. 6).

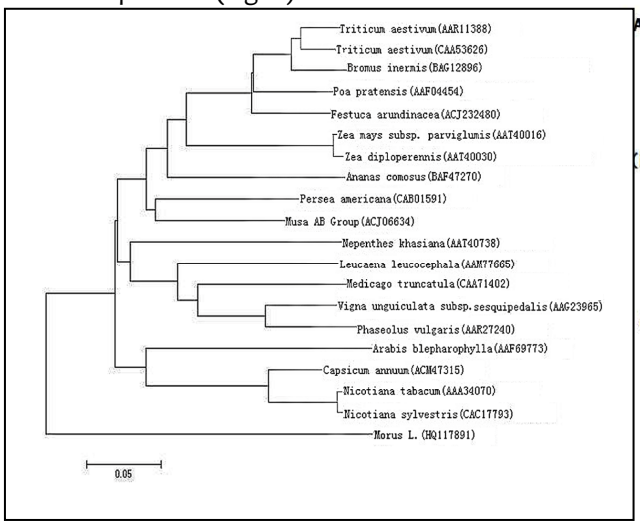


Fig.5: The phylogenetic tree based on amino acid sequence of *M-chitinase* and other homologues sequences

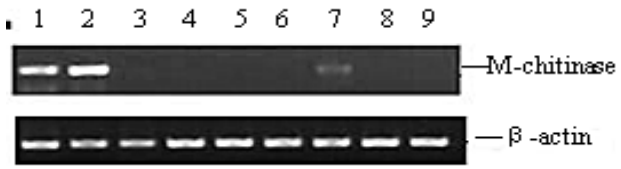


Fig.6: Analysis of M-chitinase expressing in different tissues
1. top bud;2. young leaf;3.mature leaf;4.phloem;5.xylem;6.root;7.full bloom;8.wilting flower; 9. mature fruit

Stress-induced expression patterns of M-chitinase: To further investigate whether the expression of M-chitinase was induced by abiotic stresses, we first monitored the mRNA transcription level of the *M-chitinase* gene under different abiotic stress treatments, including NaCl, SA, and ABA, by semi-quantitative RT-PCR until the appearance of symptoms.

After the treatment of salt at 6h and 12h, the expression level of the M-chitinase transcripts was to drop, and increasing at 24h then had the highest expression level at 11d, however, descending quickly at 16d and 18d (Fig. 7A). As shown in Fig. 7B, the expression level of the M-chitinase transcripts under SA stress increasing obviously and reached the highest level at 6h and 24h. Under the stress of ABA, the expression level of M-chitinase increased gradually to maximum at 12h, and then decreased. These results revealed that M-chitinase was constitutively expressed under non-stress conditions, while its expression level increased or decreased by the different degree when the mulberry was under the abiotic stress including SA, NaCl and ABA.

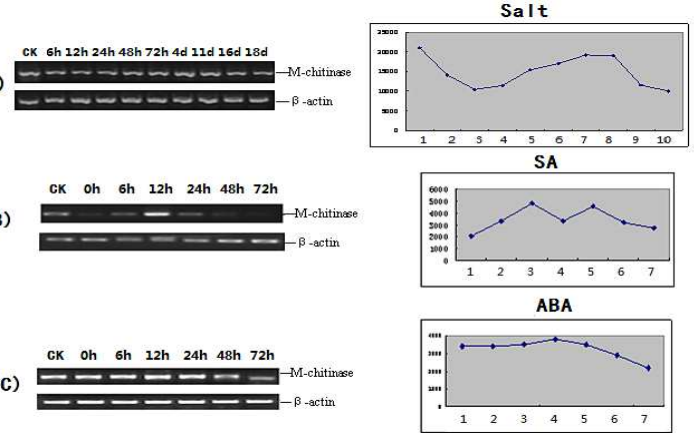


Fig.7: (A): Analysis of *M-chitinase* expressing after treatment of salt; (B): Analysis of *M-chitinase* expressing after treatment of SA; (C): Analysis of *M-chitinas* expressing after treatment of ABA

DISCUSSION

Above all, *M-chitinase* gene encoding chitinase protein was cloned from the mulberry varieties Yu71-1 (*Morus multicaulis*) and isolated from the mulberry for the first time. Full length of M-chitinase gene is 1392bp and contains a 60bp 5'-UTR (untranslated region) and a 255bp 3'-UTR. The opening reading frame (ORF) is of 1077bp, encoding 358 amino acids with a predicted molecular weight of 38.52KDa, and an isoelectric point of 4.466.Homologous alignment analysis of the M-chitinase gene proved it has very high conservatism with other species.

Earlier studies carried out by semi quantitative RT-PCR analysis had showed that young leaves had highest expression, and the mRNA transcriptional level of the

M-chitinase gene changed significantly under the conditions of NaCl, SA and ABA stresses respectively compared to the normal growth environment.

Since 1986, Broglie obtained Chitinase gene for the first time and reported the antifungal ability of the chitinase transgenic plant in 1991. And in 1987, Shin constructed the cDNA library of *Nicotiana tobacum*. Then scientists transferred Chitinase gene into *Populus nigra Linn* (Meng *et al*., 2004), *Brassica campestris L.* (Lan *et al*., 2000), *Solanum tuberosum* (Nan, 2006), *Citrullus lanatus (thunb.) mansfeld* (Wang *et al*., 2003), *Oryza sativa L.* (Nishizawa *et al*., 1999) and so on. Results showed that the antifungal ability of the transgenic plant increase in different degree. Compared with normal plant, transgenic plant not only antifungal but also has resistance of wireworm and insects. So, if we can get some useful message of chitinase transgenic plant, especially in mulberry, we can do more.

In summary, based on the results presented in this paper, it is reasonable to expect that M-chitinase most likely acts as an important common component responsive to abiotic stresses in mulberry. Further studies are underway for a more detailed elucidation of the biological function of *M-chitinase* gene, especially in the signal transduction between biotic and abiotic stress signaling. We got more useful message of *M-chitinase* gene and how the gene did in stress-induce of mulberry in this study. But the exact function of M-chitinase genes and encoded proteins in the stress response is still not fully understood in mulberry. Therefore, efforts to investigate the molecular adaptation mechanisms to stresses and to strengthen stress tolerance in plant are of fundamental importance to mulberry production.

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