Plant Omics Journal POJ

POJ 8(2):183-189 (2015) ISSN:1836-3644

Identification and expression analyses of chitinase genes in mulberry (*Morus* L.) plants

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Abstract

In the present study, we identified chitinase genes in the genome of mulberry for the first time. The mulberry genome encodes 20 chitinase genes, which are grouped into two main families and organized into five classes. The genomic structures and phylogenetic relationships of mulberry chitinase genes were analyzed, which provided a genetic basis for understanding the functions of these genes. We further investigated the expression of mulberry chitinase genes in five different tissues including root, bark, bud, flower, and leaf. Our results showed that there was no correlation between the spatial expression patterns of chitinase genes and their classification based on the conserved domains. Furthermore, six mulberry chitinase genes were used to detect transcriptional differences in their response to insect wounding, fungal infection, and biochemical elicitors. We found that different mulberry chitinase genes were induced by insect wounding and fungal infection, suggesting that these chitinases help the plant to cope with the challenges from insects and fungi.

Keywords: Chitinase; mulberry; gene expression; chitin; immune responses.
Abbreviations: GlcNAc_β-(1, 4)-linked N-acetylglucosamine; PR_ pathogenesis-related; CBD_ chitin binding domain; PDA_ Potato dextrose agar; MS_ Murashige and Skoog; NJ_ neighbor-joining; GSDS_ Gene Structure Display Server.

Introduction

Chitin, a linear, neutrally charged polymer of β-(1, 4)-linked *N*-acetylglucosamine (GlcNAc), is the second most abundant biological polymer. It is ubiquitously found in the shells of crustaceans, insect skeletons and gut linings, and the cell wall of fungi. Chitosan, a deacetylated derivative of chitin, is less common than chitin but is more biodegradable. It is present in certain species of fungi, such as *Cryptococcus* (Baker et al., 2007) and the cyst wall of *Entamoeba* (Das et al., 2006).Chitinases (EC 3.2.1.14) are defined as glycosyl hydrolases that catalyze the degradation of chitin. Chitinases have been found in a diverse range of organisms including plants, animals, fungi, and bacteria (Hakala et al., 1993; Cohen-Kupiec and Chet, 1998; Herrera-Estrella and Chet, 1999; Roberts and Selitrennikoff, 1988). According to the amino acid sequence similarity of the catalytic domains, chitinases are grouped into families 18 and 19 of glycosyl hydrolases (Henrissat, 1991). Family 18 chitinases have been characterized in bacteria, fungi, viruses, plants, and animals, whereas members of family 19 are found almost exclusively in plants (Passarinho and de Vries, 2002). However, plant chitinases from two families do not share sequence similarity and have a different tertiary structure, suggesting that they do not share a common ancestor (Hamel et al., 1997). Based on their sequences and structures, plant chitinases are subdivided into five different classes designated as class I to V (Neuhaus et al., 1996). The chitinases in classes I, II, and IV belong to the family 19 whereas those in classes III and V are plant chitinases from family 18 (Henrissat and Bairoch, 1993; Henrissat, 1991; Neuhaus et al., 1996; Hamel et al., 1997; Passarinho and de Vries, 2002). Plants may seem to be an unusual source of chitinases especially because they do not possess chitin. Plants chitinases degrade chitin and release chitooligosaccharides, which might act as elicitors to activate plant chitinase genes in a positive feedback cycle. This is a successful immune strategy plant used against microbes and herbivores (Passarinho and de Vries 2002; Wan et al., 2008; Shibuya and Minami 2001; Stacey and Shibuya 1997; Felton and Korth 2000; Ryan 1988). Therefore, chitinases are often considered as pathogenesis-related (PR) protein and important targets for crop improvement by genetic engineering (Graham and Sticklen, 1994; Legrand et al., 1987; Van Loon and Van Strien, 1999). Mulberry (*Morus* L) is a deciduous tree and an important crop used for rearing the domesticated silkworm, *Bombyx mori*. Regular pruning of mulberry plants is required to encourage leaves growth that can feed silkworms. This practice makes it susceptible to pests and pathogens. As a result, mulberry has evolved to acquire an efficient defense system. Several chitinase genes from mulberry were cloned, and their proteins were purified to study the defense response of this plant to pathogen attack (Kitajima et al., 2013). Two chitinase-like proteins, LA-a and LA-b, were isolated from the latex of mulberry. LA-a and LA-b showed insecticidal activities after feeding on the larvae of *Drosophila melanogaster* (Kitajima et al., 2010). Class I chitinase LA-c is an acidic chitinase abundant in the mulberry laticifer with antifungal activity (Kitajima et al., 2012). Completion of the draft genome of the mulberry (Morus notabilis) provides an opportunity and will accelerate the identification and characterization chitinase genes involved in mulberry defense responses (He et al., 2013). In the present study, we identified the mulberry chitinase genes. We studied the expression of these genes in five different tissues including root, bark, bud, flower, and leaf. Furthermore, we studied the transcriptional differences in the response of chitinase genes to various biotic stresses. Our results showed that these genes were induced by insect wounding, fungal infection and biochemical elicitors,

suggesting that chitinases form an important part of the plant defense response to pathogen attack.

Results

Identification of chitinase genes in the mulberry genome

Plant chitinases are similar in their structural organization having two conserved domains: Glyco_hydro_18 or Glyco_hydro_19. In addition to the catalytic domain, family 18 chitinases may contain a chitinase insertion domain. We downloaded the HMM, documents of these two domains from pfam website using the keyword "chitinase". The resultant files were used to search for putative mulberry chitinases against the mulberry genome. NCBI BLAST and SMART searches were further employed to identify the conserved domains in protein sequences. Altogether, a total of 20 genes encoding chitinases were predicted in the mulberry genome (Table 1). We predicted 12 chitinases with the Glyco_hydro_18 domain and eight with the Glyco_hydro_19 domain. Mulberry chitinases with a Glyco_hydro_18 domain were designated *Mnchi1-12* while the remaining was named *Mnchi13-20*, depending on the location of scaffolds. The mulberry chitinase genes including *Mnchi2-3*, *Mnchi5-6*, *Mnchi8-9*, *Mnchi15-17* and *Mnchi19-20* were arranged in tandem arrays. The hypothetical signal peptides in the 14 predicted chitinase sequences were also identified. Amongst these, Mnchi11 was unique as it contained the THN domain, which is related to plant pathogenesis (Ruiz-Medrano et al., 1992). Of the eight mulberry chitinases bearing Glyco_hydro_19 domain, five (Mnchi13, Mnchi14, Mnchi16, Mnchi17 and Mnchi18) contained a chitin binding domain (CBD) for chitin recognition or binding. Mnchi13 and Mnchi14 were found to possess a glycine-rich linker between the N-terminal CBD and the catalytic domain, whereas Mnchi16, Mnchi17 and Mnchi18 had a threonine/ serine linker.

Gene structures of mulberry chitinase genes

We further characterized the structural features of mulberry chitinase encoding genes such as exon/intron positions. The relative lengths of the introns and the conservation of the corresponding exon sequences within each chitinase-encoding gene were illustrated (Fig. 1B). Among the 20 mulberry chitinase genes, 10 were intronless genes, which included nine genes with the Glyco_hydro_18 domain. *Mnchi18* was the only intronless gene with the Glyco_hydro_19 domain. The majority of the remaining 10 genes (9/10) were found to have one or two exons based on the prediction, except for *Mnchi4*, which was interrupted by 15 introns. Furthermore, we analyzed the cis-elements of mulberry chitinase encoding genes (Fig. 1A). These regulatory elements such as TCA, CGTCA motif, Box-W1, TC-rich repeats, AT-rich sequence, EIRE, and WUN-motif were observed in the promoter regions of 19 mulberry chitinase genes and were found to respond to various stresses. These elements, however, were absent in the *Mnchi12* gene.

Phylogenetic analysis

Phylogenetic analysis was carried out to investigate the evolutionary relationship among the mulberry chitinases. The mulberry chitinases formed five clades, also representing five classes (Fig. 2A). Genes bearing the Glyco_hydro_19 domain were cataloged into class I, class II, and class IV. The genes in class III and class V had the Glyco_hydro_18 domain. This classification was consistent with that reported in Arabidopsis, rice and poplar (Hamel et al., 1997; Passarinho and de Vries, 2002; Jiang et al., 2013; Xu et al., 2007). Moreover, phylogenetic studies were used to make functional predictions for placing the 20 mulberry chitinases into broader evolutionary context. Chitinases with known function from *A. thaliana* and other species were used to build a phylogenetic tree. As shown in Figure 2B, 20 mulberry chitinases, 24 Arabidopsis chitinases, and 10 from other species were grouped into five classes. Class III was found to have the most mulberry genes encoding chitinases (6 genes) whereas there was only one from Arabidopsis and three from other species.

Expression profiles of mulberry chitinase genes in five tissues

Except for *Mnchi9* gene, the other 19 chitinase genes were expressed in at least one selected tissue including root, bark, bud, flower, and leaf. Based on their expression pattern, these 19 genes were clustered into four groups (Fig. 3). Group I included three genes namely *Mnchi12*, *Mnchi3*, and *Mnchi19*, which were mainly expressed in the bark and bud. The genes in Group II showed higher expression in flowers than other tissues while the genes in Group III exhibited tissue-biased expression in leaves and flowers. The remaining three genes, *Mnchi5*, *Mnchi6*, and *Mnchi13* in Group IV were highly expressed in the root, flower and leaf tissues. Furthermore, our data showed no correlation between the spatial expression patterns of chitinase genes and their classification based on the conserved domains. However, some genes that were tandemly arranged in the same scaffold were inclined to have similar expression pattern in tissues, such as *Mnchi 5-6* in scaffold594 and *Mnchi 15-17* in scaffold235.

Biotic stress and elicitor treatments affect the expressions of mulberry chitinase genes

We studied the transcriptional differences in mulberry chitinase genes of plants exposed to insect wound, fungal infection, and putative biochemical elicitors including chitin and chitosan. Six chitinase genes (*Mnchi1, Mnchi5, Mnchi8, Mnchi14, Mnchi16,* and *Mnchi19*) representing the ones from class I - class V were selected for this experiment. The ratio of transcripts for fungal infection and silkworm biting to control samples are shown in Fig. 4. Although two genes, *Mnchi*5 and 14, showed changes in their expression level in response to stresses, their expression was repressed as compared to control. However, *Mnch*i1 was slightly induced 0.5 h after silkworm feeding. *Mnchi16* was more abundantly expressed in genes from tissues subject to silkworm biting treatments than those with fungal infection. In addition, fungal infection activated the transcription of *Mnchi8* and *Mnchi19* by 3.7- and 1.6-fold after 12 h, respectively. We further investigated the expression of *Mnchi8*, *Mnchi16* and *Mnchi19* in response to two elicitors, chitin and chitosan. *Mnchi8* transcripts significantly increased in response to chitosan treatment for 7h and 12h (Fig. 5). *Mnchi16* and *Mnchi19* genes were induced by both chitin and chitosan (Fig 5).

Discussion

Chitinases are among a group of proteins involved in plant defense response against infection and wounding. The availability of *Morus* genomic data provides a unique opportunity for identifying putative mulberry chitinase genes (He, et al., 2013). Based on the sequences and conserved domains, we identified 20 chitinase genes in the mulberry genome. The number of predicted chitinase genes in the mulberry genome was comparable to *Arabidopsis* (24), and

Table 1. Chitinase genes in the *M. notabilis* genome.

Gene Name	Accession No.	Scaffold:start:end:strand	Protein size(aa)	Signal peptide prediction	Pl/Mw(Da)	Domain
Mnchi1	Morus022978	scaffold277:14116:15216:-	366	Y	5.11 / 40553.50	Glyco_hydro_18
Mnchi2	Morus007185	scaffold299:129168:130103:+	311	Y	6.35 / 34893.01	Glyco_hydro_18
Mnchi3	Morus007186	scaffold299:138035:138943:+	302	Y	7.79 / 33988.20	Glyco_hydro_18
Mnchi4	Morus017594	scaffold355:437029:444690:+	881	N	6.56 / 96769.98	Glyco_hydro_18
Mnchi5	Morus022481	scaffold594:619080:619979:+	298	Y	6.50 / 32101.29	Glyco_hydro_18
Mnchi6	Morus022482	scaffold594:624002:624898:+	298	Y	5.36 / 32012.05	Glyco_hydro_18
Mnchi7	Morus020088	scaffold604:215479:216300:+	273	N	5.96 / 30556.42	Glyco_hydro_18
Mnchi8	Morus011486	scaffold812:334910:335812:+	300	Y	8.65 / 32714.35	Glyco_hydro_18
Mnchi9	Morus011484	scaffold812:331956:332642:+	209	N	6.55 / 22965.22	Glyco_hydro_18
Mnchi10	Morus003149	scaffold1047:94797:96065:+	422	N	8.77 / 46723.48	Glyco_hydro_18
Mnchi11	Morus020224	scaffold1333:303317:307449:	508	Y	5.26 / 54848.46	Glyco_hydro_18 THN
Mnchi12	Morus000037	scaffold8877:365:964:-	199	N	7.66 / 22077.06	Glyco_hydro_18
Mnchi13	Morus007737	scaffold131:104442:106312:+	319	Y	6.78 / 35252.80	Glyco_hydro_19
Mnchi14	Morus012010	scaffold150:78835:80964:-	318	Y	6.97 / 35097.96	Glyco_hydro_19
Mnchi15	Morus018118	scaffold235:478561:479882:+	274	Y	4.59 / 29533.57	Glyco_hydro_19 CHtBD1（S/T rich in hinge）
Mnchi16	Morus018119	scaffold235:488838:490160:+	274	Y	4.71 / 29424.53	Glyco_hydro_19 CHtBD1（S/T rich in hinge）
Mnchi17	Morus018124	scaffold235:509843:511517:+	279	Y	4.56 / 30330.71	Glyco_hydro_19 CHtBD1（S/T rich in hinge）
Mnchi18	Morus013887	scaffold498:290048:290809:+	253	N	6.42 / 27844.09	Glyco_hydro_19
Mnchi19	Morus014360	scaffold629:289955:291623:-	325	Y	7.80 / 34731.89	Glyco_hydro_19 CHtBD1（G rich in hinge）
Mnchi20	Morus014362	scaffold629:310829:312523:-	325	Y	7.38 / 34893.93	Glyco_hydro_19 CHtBD1（G rich in hinge）

*Accession numbers are from http://morus.swu.edu.cn/morusdb/. aa, amino acids. CDB represents chitin binding domain.

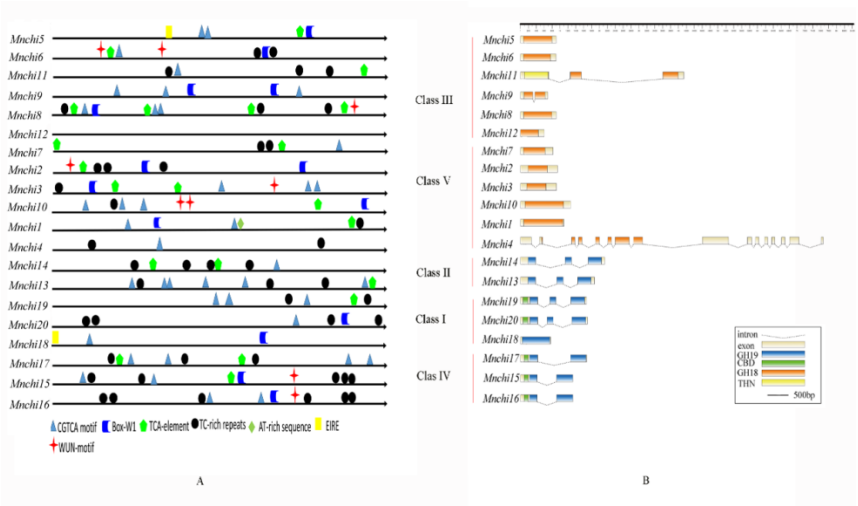

Fig 1. The genomic structures (right) and regulation elements (left) of mulberry chitinase genes. The open reading frames are marked with boxes. Colorful boxes indicate the different conserved domains. The binding sites of partial transcriptional factors are shown below the figure.

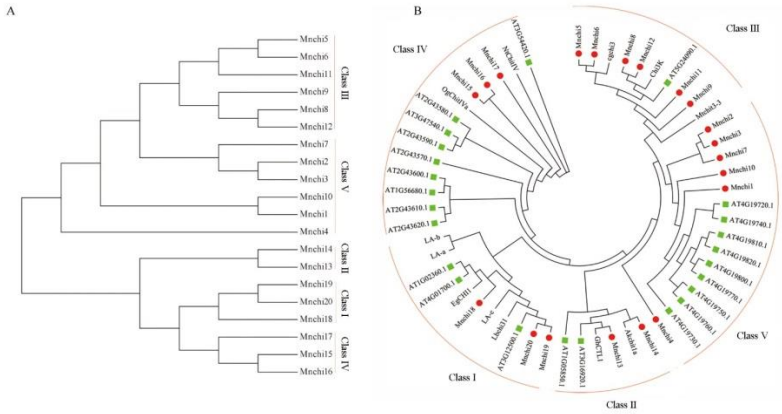

Fig 2. Phylogenetic analysis of mulberry chitinase (left) and phylogenetic tree construction of chitinases in several plants (right).
All the chitinases are grouped into Classes Ⅰ - V. The chitinases from *A. thaliana* and *M. notabilis* are marked with red cycles and green squares, respectively. Chitinases from other plants including Cgchi3 (*Casuarina glauca*); chi3k (*Vitis vinifera cv. Koshu*); Mtchit3-3 (*Medicago truncatula*); Akchit1a (*Acacia koa*); GhCTL1 (*Gossypium hirsutum*); Lbchi31 (*Limonium bicolor*); EgCHI1 (*Elaeis guineensis*); LA-a (*Morus alba*); LA-b (*Morus alba*); OgchitIVa (*Oryza grandiglumis*); and NtChitIV (*Nicotiana tobaccum*) were used.

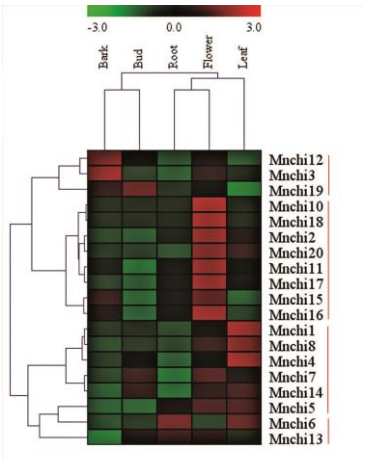

Fig 3. Expression of mulberry chitinase genes in five tissues. Expressional analysis of mulberry chitinase genes based on the RPKM profile of five tissues (root, bark, bud, flower, and leaf). Color scale indicates the degree of expression: green indicates low expression while red, high expression. Sample names are shown above the heat maps.

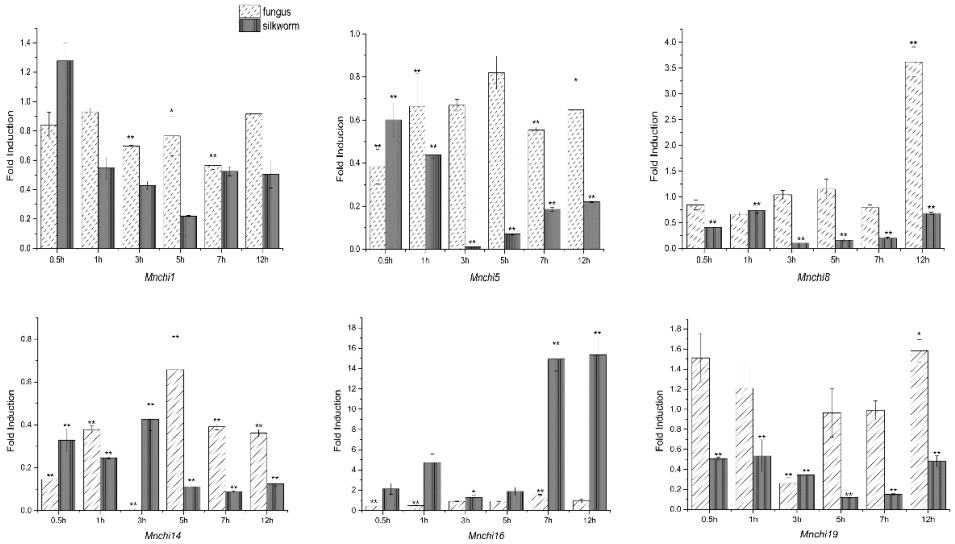

Fig 4. Expression analyses of six mulberry chitinase genes after fungal infection and silkworm feeding. Fold inductions are calculated by dividing the value of treatment with that of control in corresponding time point. Values represent the average ± SD of three biological replicates. The significance of differences are indicated by *P < 0.05 and **P < 0.01.

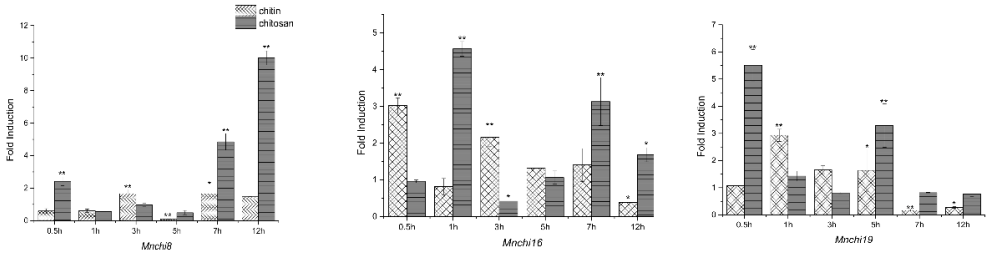

Fig 5. Expression analyses of three mulberry chitinase genes under chitin- and chitosan- treatments. Fold inductions are calculated as the method mentioned above. Values represent the average ± SD of three biological replicates. The significance of differences are indicated by *P < 0.05 and **P < 0.01.

smaller than that in poplar (37) and rice (37) (Jiang et al., 2013; Xu et al., 2007; Passarinho and de Vries 2002). Mulberry chitinase genes were divided into two families: glycosyl hydrolase encoding families, 18 and 19. Unlike the members of family 18 mulberry chitinases, five chitinases in family 19 have CBDs. Signal peptides were predicted in the N-terminal sequences of 14 mulberry chitinases. It is noteworthy that all mulberry chitinases bearing CBDs were predicted to have signal peptides. Thus, the mulberry chitinases were further divided into five classes. The chitinases Mnchi18, Mnchi19, and Mnchi20 belong to class I. Among these, *Mnchi18* is an intronless gene and its encoding protein lack CBD and signal peptide in the N-terminal, suggesting that it underwent a distinct evolutionary process. *Mnchi18* transcripts were detected in flowers, indicating that its function remains to be determined by further analysis. Class II mulberry chitinases, Mnchi13 and Mnchi14, their genes have two introns and share a gene structure similar to *Mnchi19* and *Mnchi20;* however, Mnchi13 and Mnchi14 do not have CBDs. Compared to class I chitinases, mulberry chitinases in Class IV (Mnchi15-17) have smaller molecular sizes owing to shorter CBDs and deletions of approximately 22 amino acids in their catalytic domain at the C-terminal region. The localization of chitinases depends on the presence or absence of the C-terminal extension containing vacuoles targeting information (Neuhaus et al., 1991). Therefore, the fact that these mulberry chitinases lack a C-terminal extension suggests they may be located in the intercellular spaces. We observed that there were five clusters of mulberry chitinase genes, with about 2-3 genes per cluster. These genes account for over 50% (11/20) of all mulberry chitinase genes. Thus, we hypothesized that the diversity and multiplicity of mulberry chitinase genes are generated by gene duplication. The expansion of mulberry chitinase genes seems to allow precise spatial control of their expressions due to a lack of correlation between the expression patterns and the presence of conserved domains. Chitinases play important roles in plant defense responses and their expressions are stimulated in response to insect herbivores and fungal infection (Mauch et al., 1988b, 1988a; Collinge et al., 1993; Rausher 2001; Inbar et al., 1998; Kitajima et al., 2010). Genetic manipulation of chitinase genes has shown that selection of the right target combined with transgenic technology can enhance resistance in plants and improve crop yield (Bliffeld et al., 1999; Chalavi et al., 2003; Lawrence and Novak 2006). Studies of expression patterns of mulberry chitinase genes induced by insect wounding, fungal infection, and elicitors will provide new information on the interactions between wounding and other signals and help determine the role of these genes in plant defense responses. Quantitative RT-PCR showed an increased accumulation of *Mnchi16* transcripts in the insect-wounded leaves. *Mnchi8* and *Mnchi19* were upregulated in response to *B. cinerea* infection. Our study showed that the above three stress-responsive genes could be activated by chitin and chitosan (Fig. 5). The class I chitinase Mnchi19 shares high

sequence similarity with LA-c, which was isolated from mulberry latex. LA-c was found to strongly inhibit the hyphal extension of the fungus *T. viride* and have no effect on growth of *D. melanogaster* larvae (Kitajima et al., 2012). Our observation that *Mnchi19* was upregulated in response to fungal infection corroborates Kitajima's (2012) results. Furthermore, our study showed that *Mnchi8* and *Mnchi16* were strongly induced by different factors, suggesting that different mulberry chitinases respond to different biotic stresses. Thus, our expression data together with the genetic data provides a strong basis for characterization of mulberry chitinase genes. This information will be crucial in advancing our understanding of mulberry chitinase genes for agricultural improvement.

Materials and Methods

Materials

Morus indica cv. K2 plants were grown in a greenhouse at 26 ± 2°C with a photoperiod of 14 h light and 10 h dark. *Botrytis cinerea* was cultured in a PDA (Potato dextrose agar) medium at 25± 2°C for three days. Leaves were harvested from seedlings post 3 d, 6 d, 9 d, 12 d, 15 d, and 18 d of subculture. Healthy seedlings that were propagated for 10-13 days were selected for sampling. The seedlings were first pre-incubated with fresh liquid Murashige and Skoog (MS) medium for 24 h, and then treated with 200 μg/ml chitin and 200 μg/ml chitosan for 0.5 h, 1 h, 3 h, 5 h, 7 h, and 12 h. Distilled water was used as control in this experiment. For the worm biting experiment, larvae were allowed to feed on healthy seedling leaves (4th larvae, day-3), until at least half of each leaf remained. The remaining seedling leaves were further cultured for 0.5 h, 1 h, 3 h, 5 h, 7 h, and 12 h. The treated leaves were gathered and stored at -80°C until further use.

Identification of the chitinase genes in the mulberry genome

The mulberry genomic data were retrieved from MorusDB at http://mulberry.swu.edu.cn/mulberrydb/ (He et al., 2013). The two conserved domains in chitinases were downloaded from pfam website at http://pfam.sanger.ac.uk/ using "chitinase" as query. In this website, we used the hidden Markov modeling (HMM) to identify motif characteristics of proteins at an e-value of 1e-10. The protein sequences of mulberry predicted chitinases were submitted to Simple Modular Architecture Research Tool (http://smart.embl-heidelberg.de/) and NCBI (http://ncbi.nlm.nih.gov) for further classification and confirmation (Schultz et al., 1998; Letunic et al., 2012).

Analyses of phylogeny and gene organization

Amino acid sequences of the chitinase genes in the mulberry and *Arabidopsis thaliana* genomes were aligned using Clustal X version 2 (Larkin et al., 2007). These data were used to construct a phylogenetic tree using the neighbor-joining (NJ) method (Saitou and Nei 1987). The NJ algorithm is implemented in the Molecular Evolutionary Genetics Analysis software version 5.0 (MEGA 5.0) (Tamura et al., 2011). Bootstrapping (1000 replicates) was used to evaluate the reliability of the phylogenetic tree. Exon-intron structures were analyzed using the Gene Structure Display Server (GSDS) program with default settings. The 2000 bp upstream sequences of the chitinase genes were obtained from the mulberry genome. The cis acting elements related to the defense were searched by PlantCare (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) (Lescot et al., 2002).

Expression patterns of the mulberry chitinase genes

The reads per kilobase of exon model pre million mapped reads (RPKM) were used to estimate the expression level of all predicted mulberry chitinase genes from RNA sequencing data. MultiExperiment Viewer software was used to normalize the expression levels of the chitinase genes. The heat maps illustrating the gene expression profiles were generated by Hierarchical clustering.

RNA isolation and quantitative real-time PCR (qRT-PCR)

The total RNA from samples was extracted using RNAiso Plus Kit (Takara, Japan). Reverse transcription was performed in accordance with the manufacturer's instructions (Takara, Japan). Quantitative RT-PCR was carried out on an ABI StepOnePlus real-time PCR system (Applied Biosystems, USA). Each reaction contains 10 μl 2×SYBR Green Master Mix Reagent (Takara, Japan), 2.0 μl cDNA sample, and 400 nM of gene-specific primers in a final volume of 20 μl. Primers were designed using primer premier 5.0 software and the sequences are listed in the Supplementary Table 1. Amplifications were carried out at 95°C for 30 s, followed by 40 cycles of 95°C for 5 s, 60°C for 30 s. The mulberry ribosomal protein L15 (*RPL15*) gene was used as an internal control to normalize the relative expression of target genes. The change in mRNA level for each gene after treatment was calculated as $2^{-\Delta\Delta CT}$, where $^{\Delta\Delta}CT = (^{\Delta}CT$ treatment $- ^{\Delta}CT$ control). The quantitative RT PCR analysis for each cDNA sample was repeated for three times.

Contributions

He N. and Wang X. conceived and designed research. Wang X. and Zeng Q. conducted experiments. He N. and Wang X. analyzed data. He N. and Wang X. wrote the paper. He N. and Xiang Z. revised the manuscript. All authors read and approved the manuscript.

Acknowledgments

This project was funded by the research grants from the National Hi-Tech Research and Development Program of China (No. 2013AA100605-3), the Fundamental Research Funds for the Central Universities (No.2362014xk05), the "111" Project (B12006), the Science Fund for Distinguished Young Scholars of Chongqing (Grant No. cstc2011jjjq0010).

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