Cloning and expression of class I chitinase gene from four mangrove species under heavy metal stress

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Research Article

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Abstract

Aims

Cu, Pb and Cd are common heavy metals in mangroves. Objective to clone chitinase I gene in mangrove plants and explore the role of chitinase I gene in plants under heavy metal stress.

Methods

Homologous cloning and RACE cloning were used to clone chitinase type I gene in mangrove plants, and bioinformatics analysis software was used to analyze and predict gene structure and functional domain. The mRNA expression pattern of chitinase gene in mangrove plants under heavy metal stress was analyzed by real-time quantitative PCR analysis.

Results

All Four cDNA with a full length of 1092 bp, and an ORF (open reading frame) 831 bp, coding with 276 amino acids, while are differences in the sequences among the four species. Four genes owned signal peptide and were located at vacuole inside the cell. Protein sequence domain analysis indicates that all have the same typical structural characteristics of GH19 chitinase family. The sequence of CHI had high similarity to the protein sequences of *Camellia fraternal* chitinases. Real-time PCR was used to analyze the expression of CHI under different concentration heavy metal with time of four mangrove species. The gene expression of *CHI I* was highly induced in the *B.gymnorrhiza* leaves than other mangrove species. With the increase of heavy metal stress time, the expression level of *B.gymnorrhiza* increased continuously.

Conclusion

Chitinase was induced under heavy metal in mangrove plants, and chitinase plays an active part in heavy metal tolerance in mangrove plants which was located at vacuole inside the cell.

Highlights

- Chitinase genes (CHI I) were cloned from *B.gymnorrhiza*, *K.obovate*, *A.marina* and *R.stylosa* for the first time.
- The sequence of CHI had high similarity to the protein sequences of *Camellia fraternal* chitinases.
- Protein sequence domain analysis indicated that all had the same typical structural characteristics of GH19 chitinase family.
- The gene expression of CHI I was highly induced in the *B.gymnorrhiza* leaves than other mangrove species.

1 Introduction

Mangrove wetland is an important ecosystem in the intertidal zone of tropical and subtropical coasts or at the estuary of rivers, and possesses four high characteristics of high productivity, high return rate, high decomposition rate and high resistance as one of the unique marine ecosystems in the world (Wang, 2019; Wang and Gu, 2021). It has important environmental functions and ecological benefits in wind and wave prevention, water purification, biodiversity protection, food supply and habitat (Wang, 2013; Wang, 2019). With the rapid development of modern industry, heavy metal pollution in the offshore environment is becoming more and more serious due to its toxicity and persistence in the water environment for decades. (Kamala-Kannan et al., 2008; Valls and Lorenzo, 2002). Mangrove plants also have certain tolerance to heavy metal when they live in seriously polluted environments for a long time. The adaptation

mechanisms of mangrove plants to heavy metals include: absorption and efflux of heavy metals (MacFarlane and Burchett, 1999), regionalization (MacFarlane and Burchett, 2000), chelation of organic compounds (Qin, 2007), and scavenging free radicals caused by heavy metal stress through various antioxidant defense systems (Zhang et al., 2007; Huang and Wang, 2010; Sharma and Irudayaraj, 2010) and induced expression of some defense genes (Hall, 2002; Sarowar et al. 2005; Zhang et al., 2012; Huang et al., 2010b).

It has been confirmed that chitinase is one of the related proteins (PR proteins) in plants. Plant chitinases have been divided into at least five classes (I, II, III, IV, and V) based on their sequence similarities (Collinge et al., 1993; Melchers et al., 1994). Most chitinases are also induced by some biological or no-biological factors, such as mechanical damage, chitin, ethylene, salicylic acid, heavy metals, UV, osmotic pressure, low temperature, and drought stress (Kasprzewska, 2003). In normal conditions, chitinase gene expression is very low or not expressed in most higher plants. However, when plants are infected by pathogenic fungi, bacteria or viruses, mechanical trauma or ethylene treatment, their expression activity is greatly increased, and they are often induced to express at the same time with glucanase (EC3.2.1.39), which plays an important role in plant disease resistance and defense response. Peas of three ecological types were treated with 3mg/kg Cd sand for one week, then RNA was extracted to clone stress-related genes. The results showed that chitinase gene expression was higher than that of the control (Rivera et al., 2005a). Mycorrhizal and non-mycorrhizal peas were cultured in100mg/ kg Cd sand for 3 weeks. Gene expression analysis showed that chitinase, heat shock protein, metallothionein and glutathione synthetase were significantly higher than the control group without Cd treatment (Rivera et al., 2005b). It has been reported that heavy metal ions can induce oxidative stress in plants, indicating that the accumulation of reactive oxygen species in plants under heavy metal stress leads to the accumulation of H_2O_2. The accumulation of H_2O_2 diffused and induced the transcription of chitinase gene, the accumulation of corresponding mRNA and the increase of corresponding enzyme activity (Lamb and Dixon, 1997; Fang and Kao, 2000; Tewari et al., 2002). At present, chitinase gene has been cloned from terrestrial plants, such as tobacco (Shinshi et al., 1990), potato (Ancillo et al., 1999), pear (Xiao et al., 2007), rice (Hang et al.,1991; Xu et al. 1996), etc. And studies of regulation are also deeper. However, as a defense protein, it may not directly participate in metal binding, but also plays an important role in metal tolerance of plants, which is little known. In mangrove plants, a class chitinase gene was cloned form in *A. corniculatum* (Wang et al., 2015b). It was also cloned with the full-length cDNA of a class III chitinase gene (*AmCHI III*) from *A. marina*(Wang et al., 2015a).

In the paper, the class I chitinase was firstly cloned by using RT-PCR and RACE methods in *Bruguiera gymnorrhiza, Rhizophora stylosa, Kandelia obovata, Avicennia marina.* Also elucidated for the mRNA expression pattern of CHI I in response to heavy metal stress.

2 Material And Methods

2.1 Plant material and Treatments

The six-month-old seedlings of *Rhizophora stylosa, Bruguiera gymnorrhiza, Kandelia obovata, a*nd *Avicennia marina* were purchased from Guangdong Mangrove Ecological Development Co. LTD (China). Each of the species planted 3 seedlings in each pot and divided them into 5 pots (control group CK, C1, C2, C3, C4). Each pot was irrigated with 500 mL of 1/2 Hoagland solution (containing 10% NaCl) every 3 days. The plants were watered with heavy metal sewage of different pollution levels prepared (Table1). Fresh leaves of plants were harvested after 0 day, 3 day, 7 day, 14 day, 28 day under heavy metal treatment. All the harvested samples were immediately frozen in liquid nitrogen, and stored at − 80°C before use.

Table 1
Heavy metal concentrations in artificial sewage prepared from 1/2 hogland nutrient solution.

Heavy metal (mg/L)	Control group (CK)	C1	C2	C3	C4
Cu^{2+}	0	5.0	25.0	50.0	75.0
Pb^{2+}	0	1.0	5.0	10.0	15.0
Cd^{2+}	0	0.2	1.0	2.0	3.0

2.2 Total RNA isolation and first-strand cDNA synthesis

Total RNA from leaf was extracted by centrifuging column method, using Tiangen polysaccharide polyphenol plant total RNA extraction kit (Invitrogen, USA), following the manufacturer protocol. Total RNA was dissolved into 30 μl of RNase free water. Total RNA was quantified by spectrometry, and quality was checked in denatured agarose gels. First-strand cDNA was synthesized using the PrimeScript TM Reverse Transcriptase (Takara, Dalian, China) following the manufacturer's instructions. Total RNA with 10 mM dNTP in a total volume of 20 μL by incubating for 5 min at 65ºC, 1 h at 50ºC, and 5 min at 85ºC according to the manufacturer's instruction. First-strand cDNA was stored at − 20°C before use.

2.3 Cloning the full-length cDNA of chitinase gene

The sequences of primers used were shown in Table 2. According to the conserved sequence of chitinase gene in other homologous species, the primers (F1, R1) of the intermediate fragment were designed, and the intermediate fragment was amplified. To obtain a full-length cDNA, two gene-specific primers (GSP1, GSP2) and two nested PCR primers (NGSP1, NGSP2) were deduced from the internal cDNA fragment. Then 5' -and,3' -RACE (rapid amplification of cDNA ends) reactions and PCR procedures were performed using the SMARTer TM RACE Kit (Clontech, USA) according to the manufacturer's instructions.

Table 2
List of primers for PCR, RACE, Real-time PCR experiments

Primers	Sequence (5’ – 3’)
F1	GGCTCCTTCACTTATTCACG
R1	ATTGTCTCCCCAAACCCT
GSP1	ATTGTCTCCCCAAACCCT
GSP2	GCTCCTTCACTTATTCACG
NGSP1	GCAAGAGTGAGAGATAGCGAAGGTT
NGSP2	GATACAACTGTCCTGGAACTT
qF	GTGGCACAGGCAGTGAATAC
qR	CCTTCCCCTCGCAACTAG
Bg18S(F)	CGGGGGCATTCGTATTTC
Bg18S(R)	CCTGGTCGGCATCGTTTAT
Ko18S(F)	CCTGAGAAACGGCTACCACATC
Ko18S(R)	ACCCATCCCAAGGTCCAACTAC
Am18S(F)	CCCGTTGCTGCGATGAT
Am18S(R)	GCTGCCTTCCTTGGATGTG
Rs18S(F)	ACCATAAACGATGCCGACC
Rs18S(R)	CCTTGCGACCATACTCCC

2.4 Bioinformatic analysis

The full-length sequence was subjected to bioinformatics analysis using the following software or online tools (Table 3), validation of the full-length sequence and analysis of the functional domains.

Table 3
Methods and the website of bioinformatics analysis

Function	Tool
Spliced sequence alignment.	ApE software
Predict the open reading frame	ORF-Finder (https://www.ncbi.nlm.nih.gov/orffinder/)
Predict the molecular weight, theoretical pI and hydrophilia	ExPASy-Compute-pI/Mw (http://web.expasy.org/compute_pi/)
Physical and Chemical Properties of protein	ExPASy-ProtParam (http://web.expasy.org/protparam/)
Hydrophilia	ExPASy-ProtScale (http://web.expasy.org/protscale/)
Functional site prediction of amino acid sequence	SoftBerryPSITE (http://www.softberry.com/berry.phtml?topic= psite&group=programs&subgroup=proloc)
Protein transmembrane domain analysis	TMHMM-Server-v.2.0 (http://www.cbs.dtu.dk/services/TMHMM)
Predict potential signal peptide cleavage site	SignalP4.0-Server (http://www.cbs.dtu.dk/services/SignalP-4.0)
Analysis of protein structure and function domain	SMART (http://smart.embl-heidelberg.de/)
Prediction of protein secondary structure	SOPMA(https://npsa-prabi.ibcp.fr/cgi-bin/secpred_sopma.pl)
Automated 3D structure building	ExPASy-SWISS-MODEL (https://www.swissmodel.expasy.org/)
Multiple sequence alignment	ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/)
Reconstruct phylogenetic tree	MEGA7
Predict the subcellular localization	ESLPred(http://www.imtech.res.in/raghava/eslpred/)

2.5 Analysis of *CHI I* gene expression by real-time quantitative PCR

Real-time RT-PCR reactions were performed on heavy metal from three replicates in leaf and stem tissue per treatment, performed in twofold replicates for each sample. All data are given in terms of relative mRNA expressed as the mean ± SD. The Student–Newman–Keuls test ($P<0.05$) was used to evaluate differences between means of treatments using SPSS 18.0 software. A significant difference is indicated by an asterisk ($P<0.05$).

3 Results

3.1 The full - length cDNA of *CHI I* gene cloning

As shown in Figure 1A, obvious 28S and 18S bands were observed, indicating good RNA integrity. The UV detection results showed that the OD 260 / OD 280 ratios of the total RNA samples were between 1.8 and 2.2, indicating that the RNA purity was high. Therefore, the proposed RNA has high purity and good quality, which meet the quality requirements of subsequent experiments. With leaf cDNA as the template, a specific fragment of more than 750 bp was obtained by amplification with degenerate primers (Fig. 1B). Blast was performed after sequencing, and the results showed that the fragment was highly homologous to the chitinase gene of other plants (84.73% – 74.72%), indicating that the fragment was the intermediate fragment of *CHII* gene. According to the amplified intermediate fragment

sequence, two pairs of primers for rapid amplification of 3' and 5' ends were designed. After the first and second cycles of 3' and 5' RACE PCR, the 3' and 5' end-specific fragments of the gene were amplified (Fig. 1C). Sequencing results of these fragments were spliced by MEGAE software, and submitted to NCBI for Blast homology analysis. Finally, we confirmed a correctly encoded nucleotide sequence.

3.2 Sequence and structure analysis of the full-length cDNA sequence of Chi I

Cloning and characterization analysis of the full-length cDNA sequence of cDNA sequence analysis indicated that the full-length cDNA fragment encodes a novel basic chitinase gene, designated *BgChi, KoChi, AmChi, RsChi* (Fig. 2). Four cDNA all were 1092bp, including 831bp open-reading frame encoding a protein of 276 amino acids. There are different sequences among the four species with 6–30 bases different. *BgChi* with a predicted molecular mass of 29.50 kDa and a pI of 4.47 (Table 4). *KoChi* with a predicted molecular mass of 29.59 kDa and a pI of 4.74 (Table 4). *AmChi* with a predicted molecular mass of 25.57 kDa and a pI of 4.66 (Table 4). *RsChi* with a predicted molecular mass of 29.47 kDa and a pI of 4.65 (Table 4). And the Chi I polypeptide is hydrophilic based on the hydrophilicity values (Table 4).

Table 4
Physical and chemical properties of Chi I

Species	Name of gence	Number of amino acids	Molecularweight	pI	The high content of amino acid	Instability index	Stable or not	Grand average of hydropathicity
Rhizophora stylosa	*Rs Chi*	276aa	29.47 kDa	4.65	Gly11.2% Ala9.1% Ser 9.1%	27.79	Y	-0.186
Bruguiera gymnorrhiza,	*Bg Chi*	276aa	29.50kDa	4.74	Gly11.2% Ser 9.1% Ala9.1%	27.79	Y	-0.189
Kandelia obovata,	*Ko Chi*	276aa	29.59 kDa	4.69	Gly11.2% Ser 9.1% Ala8.7%	27.16	Y	-0.218
Avicennia marina	*Am Chi*	276aa	25.57 kDa	4.66	Gly10.9% Ser 9.1% Ala8.3%	28.75	Y	-0.155

The predicted protein all had a structure typical of class I chitinases, consisting of a putative signal peptide region at its N-terminus (amino acid 1-29), a chitin-binding domain (CBD) (amino acid 31-62), and a glycosyl hydrolase catalytic domain (amino acid 76-276) (Fig. 2).

A comparison of the CHI I amino acid sequence of proteins in the GenBank database revealed that CHI I shared a high degree of similarity to the Class I chitinases of other plants (85.11% -76.95% similarity). *BgChi* showed very close homology to *KoChi* and *RsChi* in Fig. 3&4. Furthermore, the protein was predicted to be intracellular according to Plant-mpLoc (Chou and Shen, 2008). Based on SWISS-MODEL (Schwede et al., 2003) analysis, ribbon cartoon and space-filling models of CHI I were presented in Fig. 6. The GH 19 chitinase from rice (*Oryza sativa*, SMTL id: 3iwr.i.A) (Kezuka

et al., 2010), which was determined as a modeling template (Fig. 5). Four 3D models of Chi all contained seven α-helix and some random coils (Fig. 5).

3.3 CHI I mRNA expression in leaf in response to heavy metal

To realize the expression patterns of CHI I induced by heavy metal stress, Total RNA was isolated from four mangrove species seedlings leaves after heavy metal stimulation. The effects of heavy metal on the expression of CHI I mRNA in leaves were presented in Fig. 6 and Fig. 7. The real-time quantitative PCR (RT-qPCR) results revealed that the expression patterns of four mangrove species were very different. Chitinases were expressed in *B.gymnorrhiza*, *K.obovate* and *A.marina* under heavy metal stress. Under heavy metal stress, the gene expression of CHI I was highly induced in the *B.gymnorrhiza* leaves. While the expression level of *R.stylosa* was basically 0. The highest gene expression of *B.gymnorrhiza* was 55.23 times that of the control group. The highest gene expression level of *K.obovate* was 10.17 times that of the control group. The highest gene expression of *A.marina* was 14.36 times that of the control group. With the increase of heavy metal concentration, the gene expression of *B.gymnorrhiza* increased first and then decreased. The gene expression of *K. obovate* increased with the increase of heavy metal concentration.

After 3 days of heavy metal stress, CHI I gene expression was first induced in *B.gymnorrhiza*. After 7 days of heavy metal stress, the expression of *A.marina* was the highest. After 28 days of heavy metal stress, the expression of *B.gymnorrhiza* was the highest. With the increase of heavy metal stress time, the expression level of *B.gymnorrhiza* increased continuously, and the gene expression level of *K.obovate* remained stable.

4 Discussion

4.1 Cloning and structural characterization analysis of *CHI I*

Plant chitinase precursors generally contain a N-terminal signal region, a catalytic region and a C-terminal extension region. Some have chitin-binding do-rich (CBD) in cysteine after the N-terminal signal region, which is connected with the catalytic region by the variable cross-linking region (Graham and Sticklen, 1994). The GH19 family consists of all I, II and IV chitinases (Santos et al., 2008). In this study, chitinase genes (*CHI I*) were cloned from *B.gymnorrhiza*, *K.obovate*, *A.marina* and *R.stylosa* for the first time(Fig. 2). Using SMART software to predict chitinase protein structures including signal region, CBD and GH19 chitinase family catalytic domain. Most of them are small molecular proteins with molecular weight between 25 and 35 kDa (Arakane and Koga, 1999). In our study, we found that all four cDNA were 1092bp, including 831bp open-reading frame encoding a protein of 276 amino acids with molecular weight between 25.57 and 29.59 kDa (Table 4). As a result, bioinformatics analysis revealed that *BgChi, KoChi, AmChi, RsChi* were a typical class I chitinase with the characteristic catalytic structure of chitinase.

The sequences among the four species are different (Fig. 2&3, Table 4). Compared with *R.stylosa*, there is one amino acid difference in *B.gymnorrhiza*, five amino acid differences in *K.obovate*, and ten amino acid differences in *A.marina* (Fig. 3). *BgChi* showed very close homology to *KoChi* and *RsChi* in Fig. 4. We know that *B.gymnorrhiza*, *K.obovate* and *R.stylosa* belong to the same family of *Rhizophora*, while *A.marina* belongs to *Verbenaceae*. Phylogenetic tree analysis indicated that CHI had the closest relationship with chitinase in *Camellia fraternal* (75.05% similarity) (Fig. 8). Phylogenetic clustering results are more consistent with the traditional morphological classification results. *CHI I* of *A.corniculatum* exhibited very close homology to the class I chitinase from *Camellia sinensis* (69% similarity) (Wang et al., 2015a). *Camellia fraternal* and *Camellia sinensis* are similar species. *A.corniculatum* is one species of mangroves.

Iseli et al. studied class I chitinase genes in tobacco suggesting that CBD is not catalytic and antifungal activity is necessary, but binding chitin is necessary and has enhanced antibacterial effect (Iseli et al., 1993). CBD of class I

chitinases that acted as allergens in avocados and chestnuts may be associated with allergic reactions (Blanco et al.,1999). Therefore, we suppose that some of the cysteine residues in CHI I are essential for metal homeostasis in a harsh environment such as heavy metal stress.

Chitinases in plants are encoded by single genes. Both secreted outside and localized inside. In this study, the CHI protein was predicted to locate on vacuoles in cells according to Plant-mpLoc (Chou and Shen, 2008). The CHI protein was a hydrophilic protein, so we speculated about the possibility of transmembrane.

4.2 Expression of *CHI I* in leaves in response to heavy metal

Plant chitinases are induced by a series of abiotic stresses, including osmotic stress, salt stress, low temperature stress, mechanical damage and heavy metal stress (Loon et al., 2006). Class I chitinase gene was induced by mechanical damage in *Ficuscarica* (Kim et al., 2003). Chitinase genes in *Viciafaba*, *Barley*, *Maize* and *Soy bean* were induced by lead, arsenic and cadmium, indicating that this enzyme could prevent heavy metal toxicity (Békésiová et al., 2008; Keulen et al., 2008). In our experiment, in addition to *R.stylosa*, the CHI of the other three mangrove plants were induced by heavy metal stress. In terrestrial plants, the effects of chitinases have been studied to varying degrees. Stress associated proteins, like peroxidase and chitinase were also found associated with Hg in the vines (Spisso et al., 2018). CHI has chitinase activity, and may be involved in the decomposition and metabolism of the cell wall macromolecule catabolic process and carbohydrate metabolic process (Spisso et al., 2018). Plant chitinases not only play a role in metal metabolism, but also in detoxification of excess heavy metals. Heavy metal accumulation can disturb the absorption and distribution of large amounts of elements and trace elements in plants and cause plant death. Due to long-term environmental selection and adaptive evolution, plants have developed tolerance mechanisms to reduce or avoid heavy metal toxicity (Zhang and Shu, 2006). Cd treatment could induce up-regulation of chitinase, heat shock protein (HSP70) and other genes (Rivera et al., 2005a). Mycorrhizal peas and non- Mycorrhizal peas after three weeks of culture on 100 mg / kg Cd sand, the gene expression analysis showed that chitinase, heat shock protein, metallothionein and glutathione synthase were significantly higher than those in the control group without Cd treatment (Rivera et al., 2005b).

Heavy metal Cu, Cd and Pb are important pollutants in the environment, and often exist in nature compound pollution (Gu, 2003). Under combined pollution, the tolerance mechanism of plants is more complex, and it is more necessary to study the effect of combined pollution on plants and the response of plants to combined pollution. The results of this study showed that the expression of CHI I was significantly induced in leaves of *B.gymnorrhiza*, *K.obovate* and *A.marina* under combined heavy metal. Real-time quantitative results can be obtained four mangrove expression patterns are not the same under heavy metal stress. The maximum expression levels in leaves *B.gymnorrhiza*, *K.obovate* and *A.marina* were 55.23, 10.17 and 14.36 times that of the control, respectively. The gene expression of CHI I was highly induced in the *B.gymnorrhiza* leaves than other mangrove species. With the increase of heavy metal stress time, the expression level of *B.gymnorrhiza* increased continuously. *R.stylosa* is tolerant to heavy metal and has an antioxidant enzyme system(Zhou et al, 2021), while chitinase has little effect. Chitinases are located in vacuoles in cells and have the possibility of transmembrane. Chitinase expression was induced after heavy metals entered the leaves, and the expressed proteins may act on metal transporters in the cell membrane to reduce the absorption of heavy metals. That may reduce the accumulation of heavy metals on cell damage (Fig. 9). Mangrove more tolerable to the heavy metal and it can be used as a potential phytoremediator in heavy metal polluted marine wetlands.

5 Conclusion

A new chitinase (CHI) gene was cloned from *Bruguiera gymnorrhiza, Rhizophora stylosa, Kandelia obovata, Avicennia marina*. And structure includes signal peptide region at its N-terminus, a chitin-binding domain (CBD), and a glycosyl

hydrolase catalytic domain. *CHI I* belongs to glycosidase family 19. The chitinase gene sequences of four mangrove species were different. *CHI I* transcripts differentially express in four mangrove species under heavy metal. The gene expression of *CHI I* was highly induced in the *B.gymnorrhiza* leaves than other mangrove species. This study will provide more details on the molecular mechanisms or a scientific basis for coastal wetland with heavy metal environmental remediation with mangrove plants.

Declarations

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Credit Author Statement

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Declaration of Interest Statement

Conflict of Interest: All authors declare that they have no conflict of interest in this study.

Declaration of Interest: The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Figures

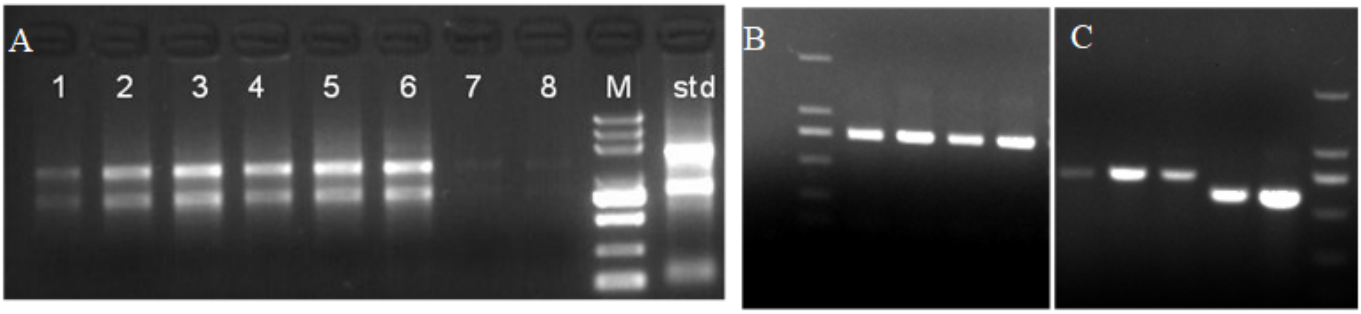

Figure 1

Agarose gel electrophoresis of total RNA (A), CHI I fragment (B), and PCR products of 3’ or 5’ RACE(C). M: DNA Marker DL5000.

Figure 2

The nucleotide sequence of the full-length cDNA and the deduced amino acid sequence of the CHI I gene. The start codon ATG and stop codon TAG are shown in bold italic, the predicted amino acid sequence is shown in one-letter code under the DNA sequence. The AATAA-box is highlighted in gray, the poly (A) tail is highlighted in gray (A: *BgChi*, B: *KoChi*, C: *AmChi*, D: *RsChi*)

Figure 3

Multiple alignment of the amino acid sequences of four Chi I protein

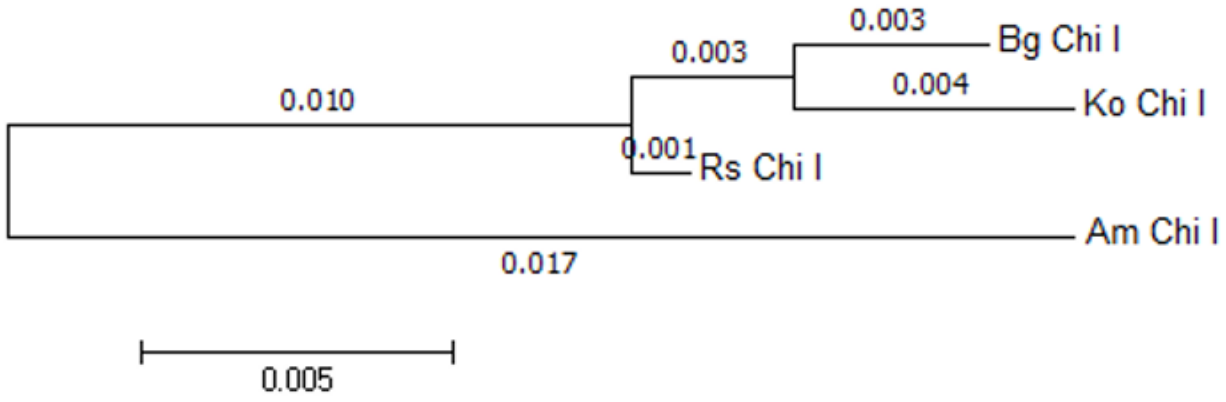

Figure 4

Similarity and phylogenetic analysis of amino acid sequence of four species chitinase genes

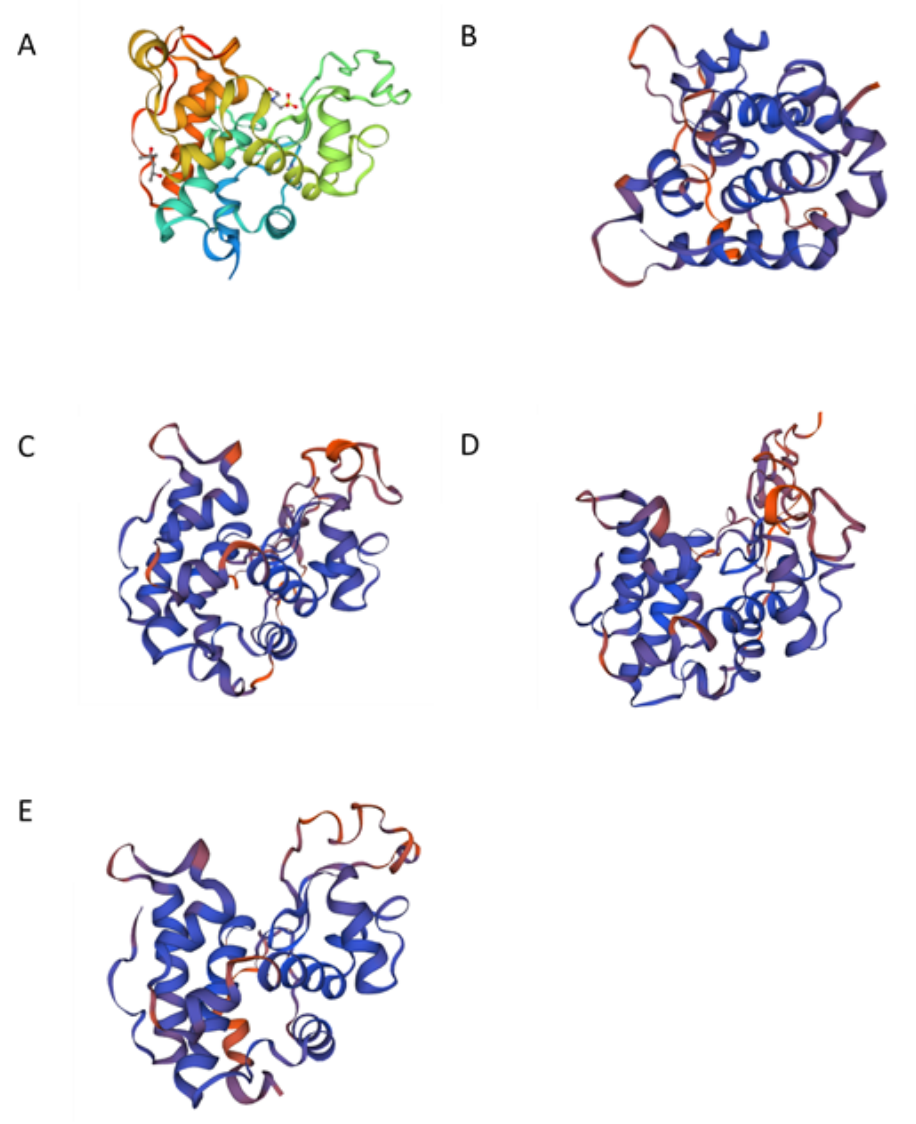

Figure 5

The molecular model of CHI I (A: *Oryza sativa*. B: *Bruguiera gymnorrhiza.* C: *Kandelia obovate.* D: *Avicennia marina.* E: *Rhizophora stylosa*).

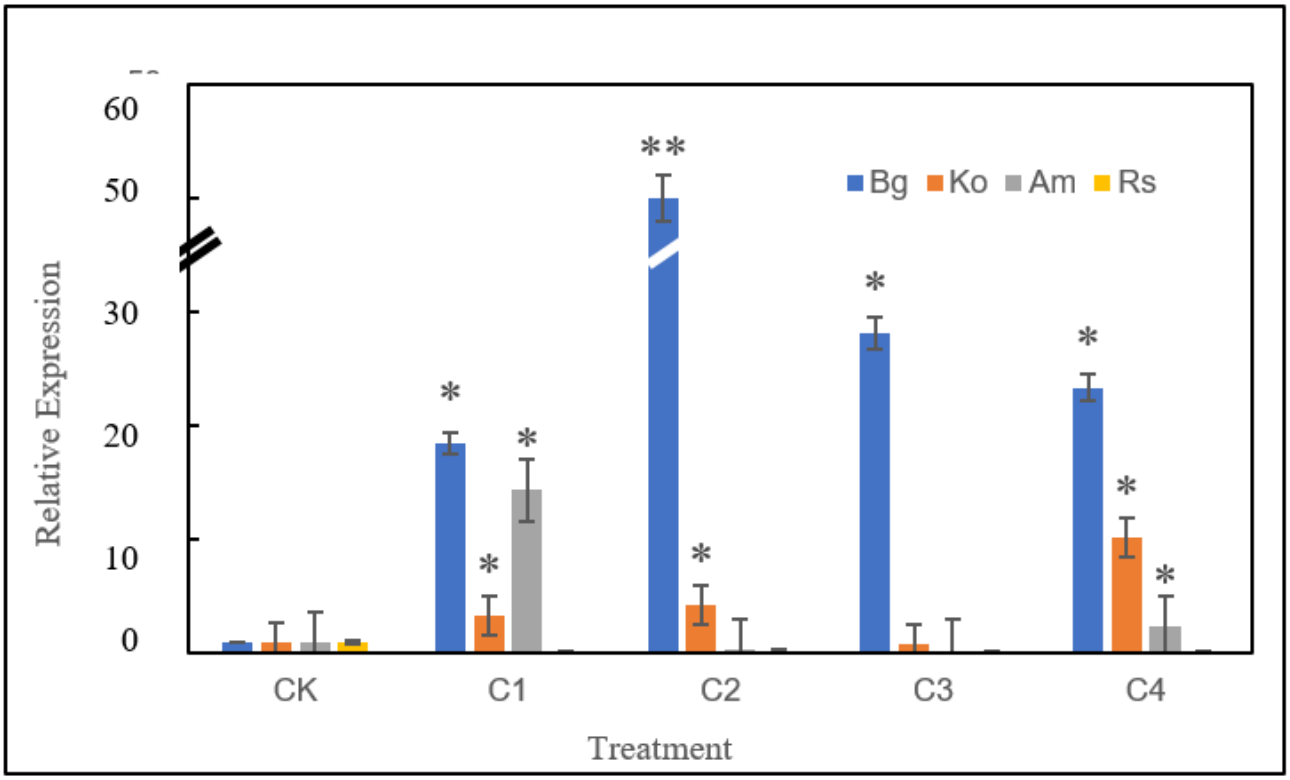

Figure 6

Expression of CHI gene in leaves of four species in response to heavy metal stresses using real-time quantitative PCR analysis. Significant difference is indicated by an asterisk ($P < 0.05$).

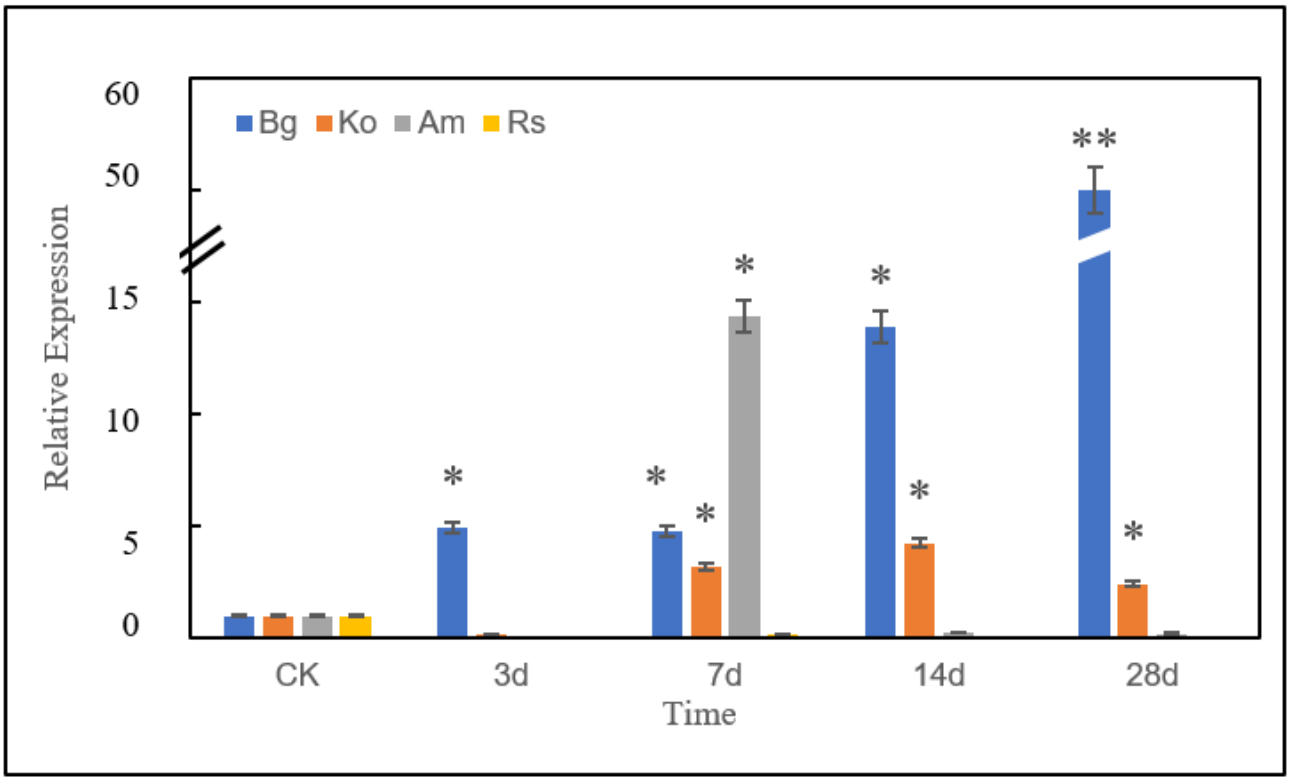

Figure 7

The time effects of heavy metal exposure on the expression of CHI I mRNA in leaves of four mangrove species. Significant difference is indicated by an asterisk (P < 0.05).

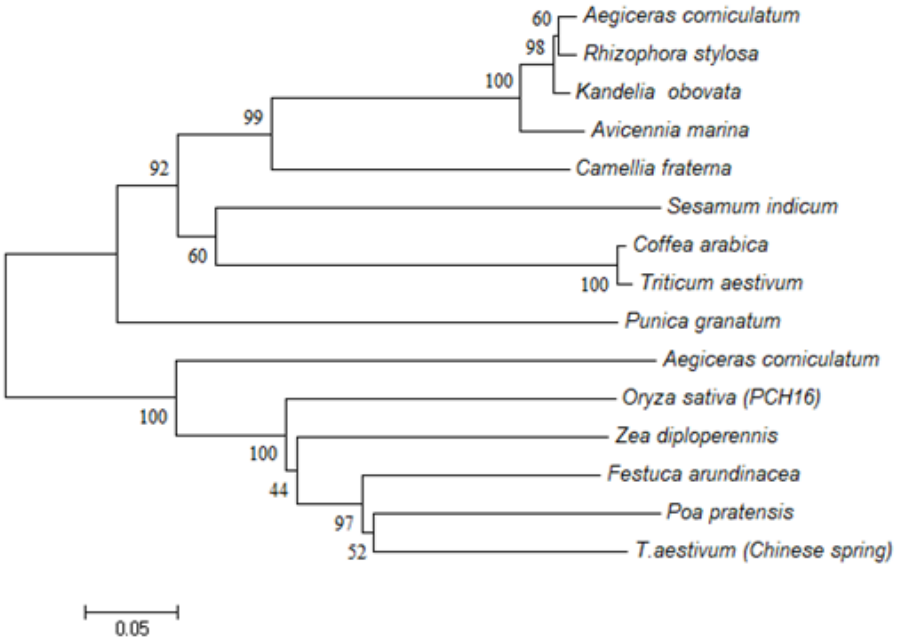

Figure 8

Phylogenetic tree of the *CHI I*. Multiple alignments of the sequences of *CHI I* and other selected plant chitinase-1 were performed using MEGA 6.

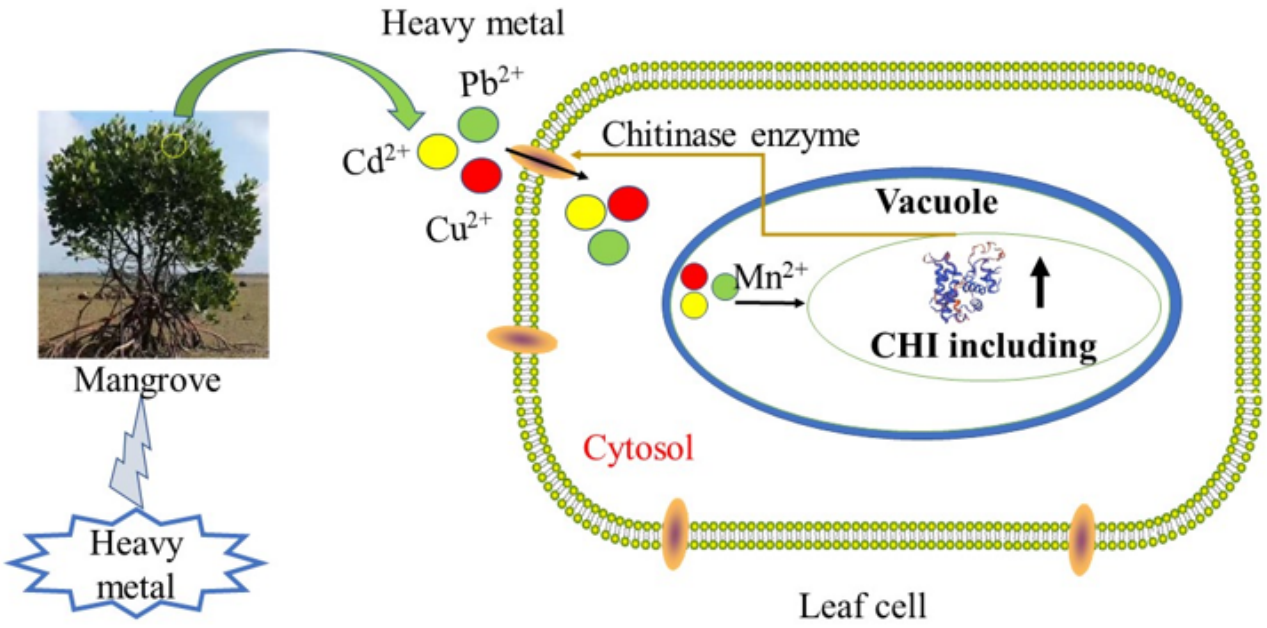

Figure 9

A schematic diagram of the mechanism of chitinase resistance to heavy metals.