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Molecular Characterization of Plantain Class I Chitinase Gene and its Expression in Response to Infection by *Gloeosporium musarum* Cke and Massee and other Abiotic Stimuli*

Jianming Fan†, Hongbin Wang†, Dongru Feng, Bin Liu, Haiyan Liu and Jinfa Wang‡
The State Key Laboratory of Biocontrol and The Key Laboratory of Gene Engineering of Ministry of Education, School of Life Sciences, Sun Yat-sen University, Guangzhou 510275, China

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We have cloned a chitinase cDNA (*MpChi-1*) from plantain (*Musa paradisiacal L*) using rapid amplification of cDNA ends (RACE) according to a sequence fragment which we had cloned using the suppression subtractive hybridization (SSH) technique. The *MpChi-1* encodes a protein of 326 amino acids and belongs to acidic chitinase class Ib subfamily. MpChi-1 shares high identity with rice endochitinase (XP_468714) and different each other only at three residues. Homology modelling indicated these three substitutions would not change the configuration of the activity site of the enzyme. We have expressed recombinant MpChi-1 and purified by ammonium sulphate precipitation and preparative reversed phase HPLC. The recombinant protein could hydrolyse chitin and inhibit the growth of the *Gloeosporium musarum* Cke and Massee *in vitro*. Northern blot revealed that the *MpChi-1* transcripts rapidly after inoculation with *G. musarum* and maximum mRNA accumulation reached at 48 h. Jasmonic acid (JA) and salicylic acid (SA) could induce *MpChi-1* expression, while mechanical wounding, silver nitrate and osmotic stress stimulated only a slight accumulation of *MpChi-1* transcripts. Abscisic acid (ABA) could induce *MpChi-1* transcript. These results suggest the *MpChi-1* plays important role in the events of the hypersensitive reaction (HR).

Key words: chitinases gene, hypersensitive reaction, induction, plantain, recombinant protein.

Abbreviations: ABA, Abscisic acid; HR, hypersensitive reaction; JA, Jasmonic acid; *MpChi-1*, *Musa* paradisiacal chitinase; PR, pathogenesis-related; SA, salicylic acid; SSH, suppression subtractive hybridization.

Plants have evolved complex and efficient defence mechanisms and strategies to protect themselves from the numerous potential invaders present in their environments. The level of resistance capability depends upon the degree of coordination among the different defence strategies and the rapidity of the overall response (*1*). Defence reactions include cell wall reinforcement, accumulation of anti-microbiol secondary metabolites such as phytomalexins, and expression of pathogenesis-related (PR) proteins. The accumulation of PR proteins (such as chitinases and β-1,3-glucanase) is one of the best-characterized plant defence responses against pathogen attack (*2*, *3*) and plants accumulate PR proteins when the plants are treated with pathogenic fungi, salicylic acid (SA) or abscisic acid (ABA) (*1–4*).

Chitinases catalyse the hydrolysis of chitin, which is a linear polymer of β-1,4-linked *N*-acetylglucosamine residues. Chitin is a major structural component of the cell walls of many pathogenic fungi. Plant chitinases are pathogenesis-related (PR) proteins, which are believed to play important roles in plant defence against infection by pathogens. Chitinases are produced in many organisms for each biological function, such as moulting of the exoskeleton in insects and crustaceans, cell growth and division in fungi, degradation of chitin for nutrients in bacteria and self-defence in plants. Plant chitinases attack pathogens directly by degrading chitin (fungal cell wall component) to confer disease resistance. Chitinases are members of the PR proteins of plants and play a role in several vital plant processes (*5*), such as plant defence against pathogens (*6*). The activation of the anti-fungal activity of chitinase, *in vitro* and in transgenic plants, may be defensive against fungal pathogens (*7*, *8*). Transgenic plants and biological control agents offer many opportunities to manipulate genes from a variety of sources for the enhancement of host plant resistance to pathogens, respectively, against insects and other types of pests (*9*, *10*). Considerable interest in the chitinolytic enzymes has been stimulated by their possible involvement as defensive agents against chitin-containing pestiferous and pathogenic organisms, such as insects, nematodes and fungi (*11*, *12*). β-1,3-Glucanases belong to a large gene family, which has been well characterized in different plant species. They have been implicated in plant responses to

*Nucleotide sequence data reported are available in the DDBJ/EMBL/GenBank databases under the accession number(s) *AY997529* and *DN796037*.
†These two authors contributed equally to this work.
‡To whom correspondence should be addressed. Tel: 86-20-84039179, Fax: 86-20-84039179, E-mail: xibao@mail.sysu.edu.cn

environmental stress and pathogen attack, and in plant developmental processes. A plant defence-related biological function for these hydrolytic enzymes has been suggested based on their coordinated induction, together with chitinases, and their inhibitory effect on *in vitro* growth and sporulation of pathogenic fungi. Another proposed defence role of β-1,3-glucanases is the release of elicitors from pathogen cell walls for the induction of the defence response. It is also proposed that β-1,3-glucanases are involved in a programmed protection against possible pathogen attack during development. The constitutive expression of chitinase and/or β-1,3-glucanase genes in transgenic plants further supports their roles in plant defence against certain fungal pathogens, especially when they are expressed simultaneously (*13*). Furthermore, chitinase and β-1,3-glucanase may release oligosaccharides (resulting from hydrolysis of the cell walls) from the fungal pathogens. These fragments may then activate other plant defence responses (*14*). Specific recognition of pathogens often induces a hypersensitive reaction (HR), which is characterized by rapid, localized cell death at the site of infection and is believed to inhibit the spread of invading pathogens. The HR is an inducible plant response that is associated with disease resistance. In most cases, the induction of a HR arrests the invasion by the pathogen (*15*). In compatible host–pathogen interactions, the fungus participates in the host's metabolism by establishing functional haustoria in the susceptible plant cells. The full HR in infected leaves is regulated by the recognition of a series of molecular triggers. Chitinase genes also offer several opportunities for gene manipulation including the enhancement of host-plant resistance to pathogens in transgenic plants and biological control agents.

Gloeosporium musarum is the most important fungal pathogen of banana and plantain, causing severe yield losses, especially in commercial cultivars worldwide and in local consumption cultivars in some regions. After the outbreak of the fungal disease, plantain consistently showed less infection in plantain than the closely related banana. We proposed, therefore that the plantain possess a more robust defensive ability that enables them to resist and survive a fungal infection, and that the resistance is enabled *via* qualitative and quantitative variations in gene expression.

In order to reveal molecular events associated with defence responses in plantain, we have constructed a cDNA fragments library of plantain leaves highly enriched for resistance-related genes using suppression subtractive hybridization (SSH) technique (*16*). Double-strand cDNA synthesis subtractive and selective amplification were performed with the PCR-Select cDNA Subtraction Kit (Clonetech, USA) as described by the manufacturer. In the forward subtraction, the cDNA from *G. musarum* inoculated plantain leaves was used as tester, the cDNA from uninoculated leaves as driver and *vice versa* in the reverse subtraction. We obtained 450 putative-specific clones, all of which were screened a second time with dot-blot hybridization. In screening the putative clones, we selected ones in which hybridization signals obtained using the forward subtraction cDNA probe were stronger than those using the reverse subtraction cDNA probe. Dot-blot hybridization gave 35 positives from the 450 putative clones. All of the selected clones were subjected to nucleotide sequencing, interestingly, of which one has high similarity with rice chitinase gene which is known as PR gene. To look into whether this plantain chitinase gene is associated with disease resistance response, we have reported on the cloning and characterization of a novel chitinase gene (*MpChi-1*) from plantain and the induction of *MpChi-1* expression in response to biological (*G. musarum*), chemical [Jasmonic acid (JA) and SA, sodium chloride (NaCl), silver nitrate (AgNO3)] and physical (mechanical wounding) stress. Finally, the degree to which the encoding protein of the *MpChi-1* inhibited hyphal extension of *G. musarum in vitro* was also examined.

METHODS

Plant and Fungal Materials—Plantain seedlings (*Musa paradisiacal L*) were grown in commercially-available potting soil in clay pots in greenhouse under a light/temperature regime of 16 h/25°C and 8 h dark/20°C Plants were watered daily. Unless otherwise stated, seedlings used in the experiments were at the four or five-leaf stage. *G. musarum* was freshly grown on PDA (potato dextrose agar) at 28°C for one or 2 days before use. The fungi were collected by gently scraping the colonies from the PDA medium with a sterile glass rod after adding 2 ml sterile of water per 85 mm plate. The fungi were prepared in a suspension, which was adjusted to an optical density at 600 nm (OD_{600}) of 0.1. Using syringe infiltration plantain leaves were inoculated with the suspension. The treated plantain leaves were then covered with clean food wrap to maintain high humidity and transferred to a growth chamber and incubated at 25°C under continuous light. Initially small, yellow and circular spots appear on the plantain leaves, and 72 h post-inoculation the symptoms of yellowing and necrosis were clearly visible, and parts of leaves began to decay.

The mechanical wounding experiment, which was designed to determine the local wound response of the *MpChi-1* gene, was conducted according to the methods described by Cordero *et al*. (1994). Fully expanded intact leaflets were injured by punching four cuts on each side of the middle vein and incubated at 25°C under continuous light and then harvested 24 h and 48 h later. Solution of $AgNO_3$, and SA were prepared in sterile water. JA was first dissolved in ethanol, and was then brought to final volume in 0.05% (v/v) Tween-20. ABA was dissolved in ethanol (100 mM stock solution) and diluted in a 20 mM sodium phosphate buffer, at a pH of 6.0, to a final concentration of 50 μM. To investigate the *MpChi-1* responsiveness to JA (50 μmol/l), SA (2 mmol/l), ABA (50 μmol/l) and $AgNO_3$ (50 μmol/l), plantain leaves were sprayed with the particular chemical solution. For chemical treatments, leaves were treated test solutions without chemicals (controls) or treated solutions with chemicals. After 6, 12, 24, 36, 48 and 72 h, the leaf samples were harvested, weighted and frozen immediately in liquid nitrogen.

5′-and 3′-Rapid Amplification of cDNA Ends (RACE)—According to the partial sequencing result of single subtractive library clone fragment (our unpublished

data), we found that the EST008 clone (Genebank Accession number DN796036) shows high similarity to the chitinase from rice. To clone the full-length of this cDNA, 5′ RACE and 3′ RACE was performed. We designed the two gene-specific primers, GSP1 (5′ACCAGAAGGCCGTGTCGAAGG3′), GSP2(5′CGACGC CGACACCAACAAGCG3′). RACE was carried out using the GeneRacer™Kit (Invitrogen, USA). Poly(A) +RNAs extracted from young plantain leaves infected with *G. musarum Cookeet Mass* were used as templates. Reverse transcription and adaptor analysis were performed following the manufacture's protocol. The 5′-end of the cDNA was PCR-amplified in an amplification reaction (50 µl) containing 2.5 µl of 5′-RACE-Ready cDNA, 0.2 mM deoxyribonucleotides, 10 µM of a gene-specific primer GSP2 (5′CGACGCCGACACC AACAAGCG3′), 10 µM of the GeneRacer™5′ Primer in the kit and 2 U of LaTaq DNA polymerase (TaKaRa, Japan). Temperature cycling was carried out at 96°C for 3 min, followed by five cycles of 20 s at 96°C and 3 min at 72°C, five cycles of 20 s at 96°C, 30 s at 70°C and 3 min at 72°C, and 22 cycles of 20 s at 96°C, 30 s at 68°C and 3 min at 72°C. Followed by 8 min final extension at 72°C. The 3′-end of the cDNA amplification reaction (50 µl) containing 2.5 µl of 3′-RACE-Ready cDNA, 0.2 mM deoxyribonucleotides, 10 µM of a gene-specific primer GSP1, 10 µM of the GeneRacer™3′ Primer in the kit, and 2 U of LaTaq DNA polymerase (TaKaRa, Japan). Temperature cycling was carried out under the same conditions as for 5′-RACE. The PCR products were cloned into a pMD18-T Vector (TaKaRa, Japan) and the nucleotide sequences were determined as described earlier.

Overlap extension of PCR amplifying full-length cDNA Overlap extension PCR was performed as follows: diluted RACE products 10×, mixed 1 µl dilution together, added 5 µl 10× LaTaq DNA Polymerase buffer, 5 µl dNTPs (2 mmol/l), 0.5 µl *Taq* DNA polymerase (TaKaRa, Japan), 36 µl ddH_2O, denatured at 96°C for 3 min, 75°C overlap extension 20 min, immediately added 1 µl GeneRacer™ 5′ Primer and GeneRacer™ 3′ Primer and then continued by 96°C for 20 s, 68°C for 30 s, 72°C for 1.2 min, 22 cycles followed by a 5 min final extension at 72°C.

DNA Sequence Analysis—The sequences obtained were compared against the sequences in NCBI Genebank database using the online BLAST program. To predict the protein encoded by these cDNAs, a homology search of the public database was conducted using BLASTx and BLASTp program. Homology modelling was performed through HMMSTR server (*17*). Barley 1 cns was selected and used as the structural template for molecular modelling. The molecular graphics were generated with the PhMol (*18*). Protein sequences corresponding to the chitinase genes were aligned using the Clustal W Program in BioEdit (*19*). The gaps were removed and the alignments were used to construct phylogentic trees with the neighbour-joining algorithm as implemented in MEGA3 (*20*), based on Poisson distances inferred from the amino acid sequence alignments. Meanwhile, bootstrap analyses involving 1000 replications were performed.

Construction of the pET11a-MpChi-1 Expression Plasmid—Primers P1 (sense) 5′CCG**CATATG**ATG GAGCAGTGCGGCAGCCAG (*Nde*1 site underlined), and P2 (antisense) GGCT**GAATTC**ATACTCCATCAGT GATTA (*Eco*R1 site underlined) were designed for amplifying the cDNA region encoding the 34.0 kDa mature protein of 'MpChi-1' from plasmid pMD18-T Vector (T-MpChi-1) containing the *MpChi-1* cDNA and the signal peptide had been 'cleavaged'. The pET11a vector was digested with *Nde*1 and *Eco*R1, The PCR product was digested with the same enzymes which were inserted to construct the expression plasmid pET11a-MpChi-1. The DNA sequence of the mature region of pET11a-MpChi-1 was confirmed by direct sequencing (BIOSIA, Shanghai, China).

Protein Expression and Purification—The following general conditions were used for protein preparation. *Escherichia coli* BL21 (DE3) cells harbouring the expression plasmid were grown to 0.4–0.6 OD_{600} in 1L LB medium containing 100 µg/ml ampicillin. Cultures were induced to produce the target protein by addition of isopropyl-1-thio-β-D-galactopyranoside (IPTG) to a final concentration of 0.5 mM followed by additional 3.5 h incubation at 37°C. Cells were then harvested by centrifugation for 15 min at 8000 × *g* (4°C) and the cell pellet was lysed by sonication and resuspended with an equal volume of 20 mM Tris–HCL (pH8.0). The supernatant was used for enzyme purification. Ammonium sulphate was added to the supernatant at 80% saturation. The solution was kept overnight at 4°C. After centrifugation for 20 min at 8000 × *g* (4°C), the precipitate was resuspended and stirred overnight at 4°C and then dialysed successively four times with 100 volume of 50 mM Tris–HCl, (pH 8.0) at 4°C to obtain crude chitinase. The recombinant protein was concentrated by ultrafiltration on a Macrosep centrifugal device (PALL, USA) and freeze dried.

RP-HPLC Purification and Analysis—An Alliance HPLC system (HP1100, USA) consisting of an integrated controller, pump, autoinjector and a photodiode array detector was used. A pre-packed Diamonsil™ C18 HPLC column (250 × 4.6 mm, 5 µm particle size) with a flow rate of 0.8 ml/min, and a mobile phase consisting of acetoonitrile (D) and water (E), were continuously degassed. A gradient elution was performed using the following solvent gradient: from 20D/80E to 80D/20E in 20 min. UV detection was performed at 280 nm, and all samples were injected in triplicate. Chromatographic data were processed and the fractions were pooled.

The semi-purified recombinant MpChi-1 was finally purified by preparative reversed phase HPLC on Diamonsil™ C18 HPLC column using a water/acetoonitrile buffer system. The MpChi-1 containing fractions were pooled and following removal of organic solvent, lyophilized and stored at –20°C. The protein purity was monitored by analytical reversed phase HPLC using a Jupitor C18 column, and estimated by SDS–PAGE followed by staining with Coomassie blue and scanned image analysis using the Bio-Rad Quantity One software.

Chitinase Activity Assay—Chitinase activity was assayed colorimettrically using Remazol Brilliant Violet-labelled carboxymethyl-chitin (CM-chitin-RBV; 2 mg ml^{-1}, Sigma, USA) as the substrate, with a method modified from Wirth and Wolf (1992) (*21*). Each assay was

performed in a 0.5 ml microfuge tube containing 50 µl of CM-chitin-RBV, 100 µl 100 µM sodium acetate buffer (pH 5.0) and 50 µl purified chitinase samples. After incubating in a 37°C water bath for 2.5 h, the enzyme reaction was terminated by cooling the tube on ice for 10 min before 50 µl of 0.3 N HCl was added to precipitate non-degraded CM-chitin-RBV. After centrifugation (1450 *g* for 10 min at 4°C), the supernatant (200 µl) was transferred to a microcuvette and A_{550} was measured spectrophotometrically against a blank (incubation mixture without enzyme sample added).

Assay of Anti-fungal Activity—The growth of *G. musarum* was inhibited by purified chitinase. Since the anti-fungal activity appears to be a general property of the chitinases, we also determined if our recombinant chitinase inhibited the growth of *G. musarum*.

RNA Gel-Blot Analysis—Total RNA electrophoresis and the programs of the RNA transferred and cross-linked onto a nylon membrane (Hybrid N^+, Amersham, Buckinghamshire, UK) were performed as described by Ge *et al*. (2000) (*22*). Total RNA of 30 µg was loaded on each lane. The probe of 5′cDNA fragment (760 bp) of *MpChi*-1gene was labelled with [^{32}P] dCTP (China Isotope, Beijing) or with Dig labelled was synthesized for hybridization. After hybridization for 20 h at 68°C, the membrane was washed once with 2×SSC plus 0.1% (W/V) SDS at 68°C for 20 min, then washed with 1×SSC plus 0.1% (W/V) SDS at 37°C for 30 min. The membrane was exposed to the X-ray film (Kodak, Rochester, NY, USA) at –70°C for 3–7 days.

RESULTS

Cloning and Sequence Analysis of Plantain MpChi-1 Gene cDNA—To clone the full-length of chitinase gene cDNA, 5′ RACE and 3′ RACE was performed. After RACE amplification, 10 µl aliquots of the amplification products were separated on 1.2% agarose (containing EB 0.5 µg/ml) gel. The 5′-RACE product was about 769 bp and the 3′- RACE fragment was about 671 bp in length. Overlap extension PCR produced a single band of 1082-bp long. In order to verify each other, we cloned the overlap extension PCR products and the 5′and 3′RACE products. The sequencing results showed that the full-length was in accordance with the sequence analysed with 5′-and 3′-RACE products separately. The complete cDNA sequence was designated as the *MpChi-1* (Genebank accession number AY997529), which has a length of 1082 bp with an open reading frame (ORF) of 978 bp and codes putative 326 amino acids acidic class I chitinase. It has a 43 bp 5′ untranslated sequence and a 63 bp at 3′ non-coding region (Fig. 1). The putative gene product of *MpChi-1* has a calculated molecular weight of 33.58 kDa and an isoelectric point at pH 4.70.

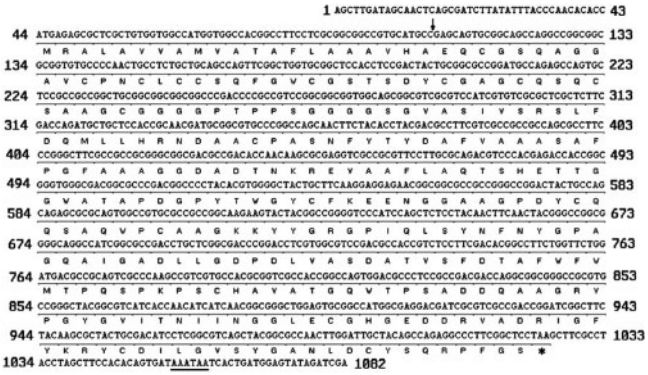
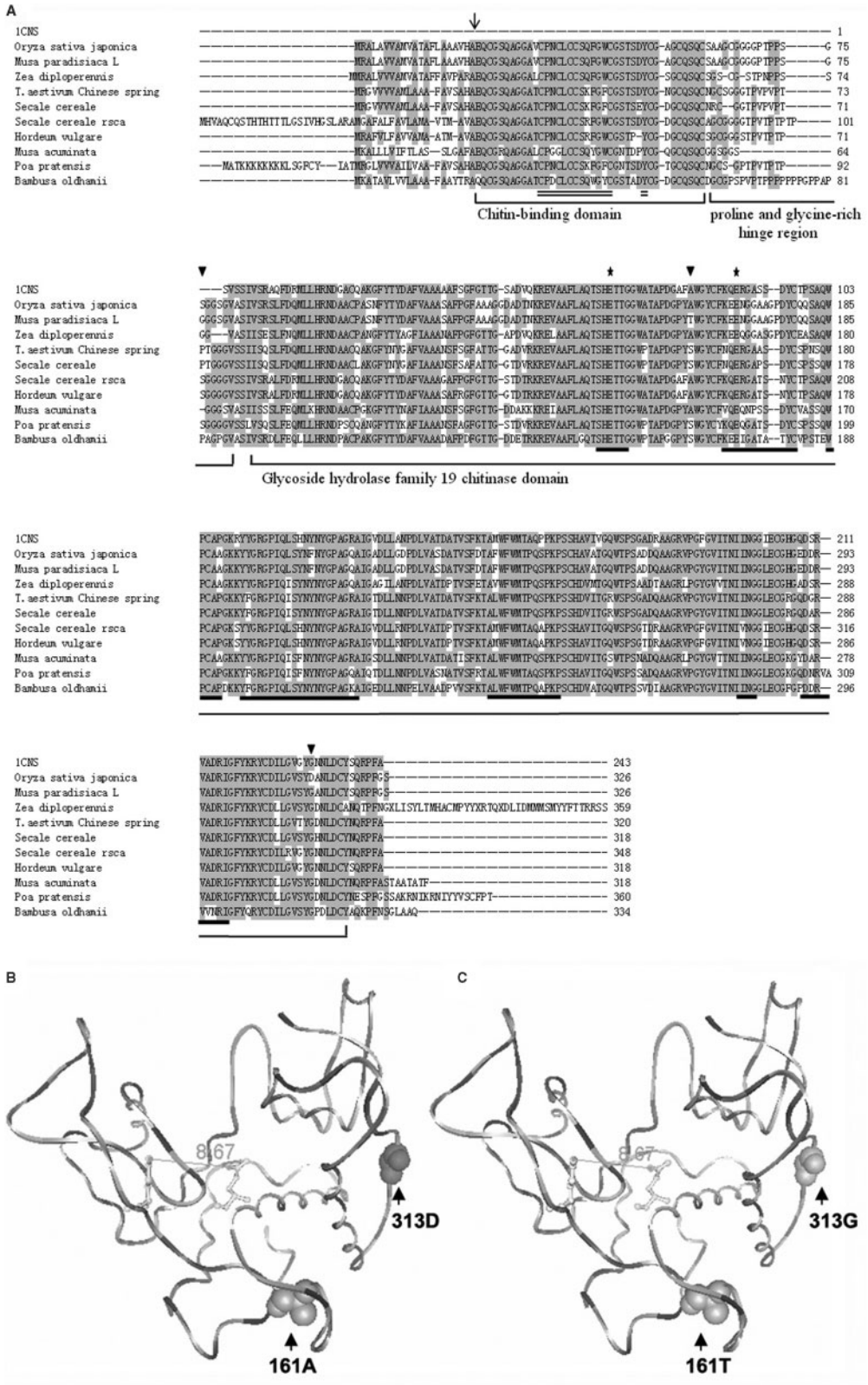


Fig. 1. **Nucleotide and deduced amino-acid sequences of *MpChi-1*.** Nucleotides are numbered in the 5′ to 3′ direction, beginning with the first residue of the putative initiation methionine codon ^{44}ATG^{46}. The predicated amino-acid sequence of *MpChi-1* is shown below the nucleotide sequence. The termination codon TAA is marked with an asterisk. A putative polyadenylation signal (AAATAA) is underlined. The predicated cleavage position of signal peptide is marked with arrow.

A search of protein databases with the deduced amino acid sequence using the Clustal W Program in BioEdit revealed that the protein encoded by *MpChi-1* shares significant sequence similarity with all other plant chitinases cDNAs. The deduced amino acid sequence contains a N-terminal cysteine-rich domain of approximately 40 amino acids and a highly conserved glycoside hydrolase family 19 chitinase domain, separated by a hypervariable proline–glycine-rich hinge region. The N-terminal cysteine-rich domain is considered as the chitin-binding domain (*23*). The N-terminal region is constituted of hydrophobic amino acids and shares similarity to known signal peptide sequences and may be cleaved from the mature peptide (*24*) However, the carboxy-terminal extension for targeting to plant vacuole is not present, suggesting an extracellular location (*25*). Together with the fact that the protein has the pI of 4.70, MpChi-1 belongs to the acidic chitinase class Ib subfamily, and locates in apoplast. Alignment of the chitinase class I in monocot shows that these proteins have highly conserved region in the chitin-binding domain and catalytic domain but not in the signal peptide and hinge region (Fig. 2A). Surprisingly, MpChi-1 and rice endochitinase (XP_468715) showed

Fig. 2. **Sequence alignment and homology modelling.** (A) Alignment of the amino acid sequence derived from MpChi-1 cDNA with sequence from barely (1cns), *Zea diploperennis* (AAT40035), *T. aestivum Chinese spring* (CAA53626), *Secale cereale* (AAG53609), *Secale cereale* (BAB18519), *Hordeum vulgare* (P11955), *Musa acuminate* (CAC81811), *Poa pratensis* (AAF04453) and *Bambusa oldhamii* (AAR18735). Amino acids marked in grey boxes indicate sequence identity. Gaps introduced for optimal alignment are indicated by dashes. The chitin-binding domain, proline and glycine-rich hinge region and glycoside hydrolase family 19 chitinase domain are labelled. The two catalytic residues are designated by an asterisk above the amino acid (*26*). The active-site residues are underlined and the three residues difference between MpChi-1 and rice endochitinase (XP_468715) are designated by triangle. Residues within chitin-binding domain relevant for chitin binding are double-underlined (*23*). Putative signal peptide cleavage site is represented by an arrow. Homology models of rice endochitinase (C) and MpChi-1 (D) of catalytic domain were generated based on barley 1cns 3D structure. The model is illustrated as a ribbon representation and the different residues between these two proteins (residues 161 and 313) are labelled.



extremely high level of sequence identity of 99%, differ only in three residues 76 (G/S), 161 (T/A) and 313 (G/D). Among the three residues, the residue 76 locates in the hinge region, only residues 161 and 313 locate in the catalytic domain. However, both these residues are not involved in the active-site regions that comprises the binding site of chitinase (*26*). According to the molecular modelling of the 3D structure of MpChi-1 and rice endochitinase base on the catalytic domain (Fig 2B and C), both protein have the substrate-binding cleft, the substitution at residues 161 and 313 will not change the distance between the two catalytic residues 147E and 169E (8.67 Å), suggesting the conservation of catalytic activity of these two enzyme.

A phylogenetic analysis of the 10 deduced chitinase amino acid sequences in Genebank was used to compare the relationship of the chitinase encoded by plantain *MpChi-1* to other monocot chitinases. Well-separated branches of plant chitinases can be identified in the phylogenetic tree (Fig. 3) and monocot chitinases of each plant are clustered together.

Characterization of the Purified MpChi-1 Protein—The cDNA region encoding the 34.0 kDa mature protein of 'MpChi-1' (the signal peptide had been 'cleavaged') was cloned into the prokaryotic expression vector pET11a as a *Nde*I/*Eco*RI fragment. A novel induced protein of ~34.0 kDa was found in induced cultures that were transformed with the recombinant pET11a-MpChi-1 plasmid. The supernatants of the sonicated cell lysates were tested for the presence of recombinant chitinase by SDS–PAGE. The primary purified recombinant chitinase was treated by preparative reversed phase HPLC (Fig. 4A). The analytical reversed HPLC indicated that recombinant chitinase was well purified (Fig. 4B). A band corresponding to ~34.0 kDa was clearly observed in the gels containing the purified chitinase using SDS-PAGE (Fig. 4C).

Chitinase Activity Assay—Using the hydrolysis of CM-chitin-RBV as the assay method, the activity of the purified MpChi-1 at 37°C and pH8 was 1.65ΔA550/μg/h, whereas that of the crude extracted enzyme was 0.21ΔA550/μg/h. The activity of the purified MpChi-1 was 7.1 times greater than that of the crude extracted enzyme.

Anti-fungal Activity of the Purified Chitinase—The inhibitory effect was observed using of the recombinant chitinase (Fig. 5). The inhibitory effect was observed using 40 μg (Fig. 5, Disk 2) and 30 μg (Fig. 5, Disk 3) but not 10 μg (Fig. 5, Disk 5) and 5 μg (Fig. 5, Disk 6) of the recombinant chitinase.

Induced Expression of MpChi-1 mRNA by Various Stimuli—Pathogen-induced expression analysis of MpChi-1

To evaluate qualitatively the MpChi-1 induction kinetics by *G. musarum*, we looked at its expression pattern during infection. Northern blotting analysis revealed that MpChi-1 mRNA was accumulated, and was initially detected 12 h post-inoculation (Fig. 6A). The substantial increase in MpChi-1 mRNA coincided with the appearance of necrosis (yellowing) of the leaves. As the evidences of necrosis increased, the MpChi-1 gene mRNA accumulation also increased correspondingly (data not shown) 72 h post-inoculation, when MpChi-1 transcripts accumulation reached the maximum level, the symptoms of yellowing and necrosis were clearly visible, and parts of leaves began to decay.

Effect of Salicylic acid (SA) and Jasmonic acid (JA) treatments on MpChi-1 mRNA accumulation

SA and JA have been implicated as the most important endogenous signals in the induction of resistance against microbial pathogens (*27*). To determine whether SA and JA can induce expression of the *MpChi-1* mRNA, total RNA was extracted from plantain leaves treated with SA and JA solution and probed with specific *MpChi-1* probe. For SA, *MpChi-1* transcripts were detected 6 h after treatment, decreased slight and then reached the high level by 36 h (Fig. 6C). The accumulation of the *MpChi-1* mRNA was detected during treatments with SA and JA (Fig. 6C and D), indicating that *MpChi-1* expression can be induced not only by JA but also by SA.

Response of MpChi-1 to wounding and osmotic stress, ABA, $AgNO_3$

We examined whether *MpChi-1* mRNA that were treated by being wounded mechanically and soak with NaCl, ABA and $AgNO_3$ solution. Accumulation of the transcript in leaves occurred at consistent levels after wounding and only slightly increased with an increase in the NaCl concentration from 0.5% to 1.5% after 48 h treatment (Fig. 6B). These data demonstrate that the expression of the *MpChi-1* could be induced by both wounding and osmotic stress, but the level of expression was higher with the former stressor. Accumulation of the *MpChi-1* transcript could also be induced by $AgNO_3$ and ABA.

DISCUSSION

Annual crop losses due to fungal caused disease and mycotoxin contamination cost billions of dollars annually worldwide. Although progress has been made in combating the disease, adequate control measures remain elusive and biological control has emerged as a promising strategy. Chitinases are enzymes that serve critical roles in fungal growth and development, in resistance of plants to fungal pathogens and in parasitism of insects by entomopathogenic fungi (*9*). The importance of chitinase as a component of a broad-spectrum plant defence mechanism has been well studied (*28–30*). Sequence analysis indicated that MpChi-1 belongs to acidic chitinase class Ib sub-family and locates in apoplast. It not only has the catalytic domain, but also has the chitin-binding domain. In modelled MpChi-1, the substrate-binding cleft and active site region present suggesting that the enzyme has the chitinase activity which was confirmed by the *in vitro* analysis with purified recombinant protein. Moreover, the anti-fungal activity of MpChi-1 also was identified by the *in vitro* assay. These results suggest that the MpChi-1 might exhibit an important role in plant defence which protect plant against pathogenic fungus colonization by destroy the chains of chitin, repressing fungal growth.

The purified recombinant MpChi-1 could inhibit the growth of the *G. musarum in vitro* (Fig. 5). Anti-fungal

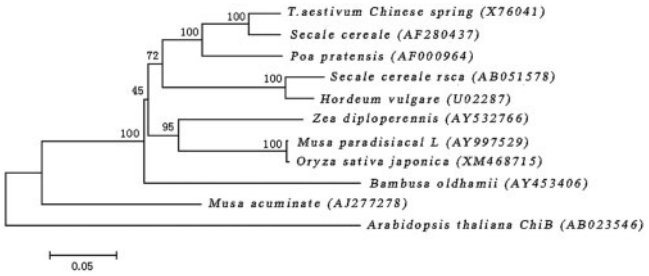


Fig. 3. **Phylogenetic analysis of the aligned amino acid sequence of plantain (*Musa paradisiacal L*) chitinase *MpChi-1* cDNA and other monocot chitinase genes.** The other chitinases are indicated by the names of the plants of origin and their Genebank/EMBL accession numbers. One *Arabidopsis* class I chitinase was introduced as the out-group. The horizontal lengths of branches are proportional to the relative homologies between chitinase sequences.

activity of chitinase can be effectively utilized in biologic pest control strategies. Therefore, our *MpChi-1* and purified recombinant chitinase will be also useful for biocontrol of fungal infection of plants in addition to chitin degradation.

Many researchers have proposed that chitinase expression may represent a prophylactic or inducible mechanism for protection against microbial invasion (*31*, *32*). In this study, fungal infection results suggested that the MpChi-1 was inducible in response to the *G. musarum* pathogen (Fig. 6A). The fact that the induction of transcripts occurs within 24 h in the response suggested that MpChi-1 is involved in defence against *G. musarum* infection and this chitinase may also be activated by signalling transduction pathways, releasing elicitors for the establishment of a HR. The induction of MpChi-1 by *G. musarum* infection, led us to further investigate its

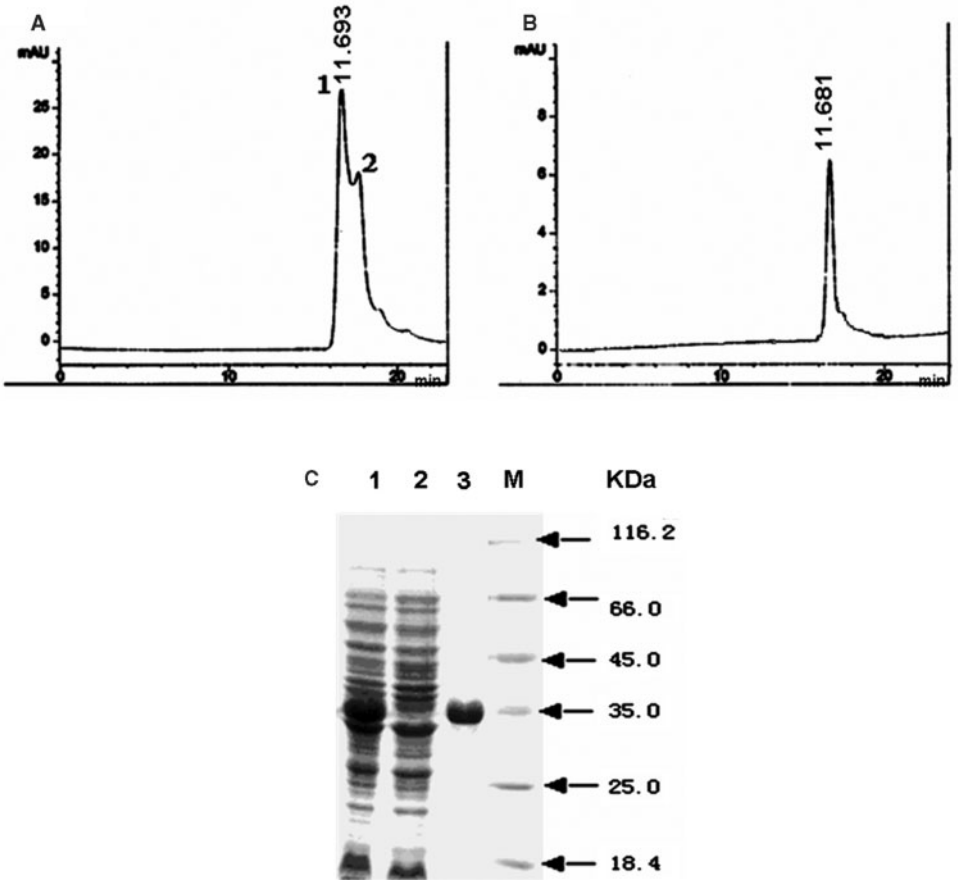


Fig. 4. **Reverse phase HPLC profiles for preparation (A) and analysis (B) of recombinant MpChi-1 and SDS–PAGE pattern of cell extracts from *E. coli* expressing *MpChi-1* and result of purification (C).** The strongest peaks in Fig. A and B represents MpChi-1, the weak peak in A represents salts and other impurities. A pre-packed Diamonsil ™ C18 HPLC column (250 × 4.6 mm, 5 μm particle size) with a flow rate of 0.8 ml/min, and a mobile phase consisting of acetonitrile (D) and water (E), were continuously degassed. A gradient elution was performed using the following solvent gradient: from 20D/80E to 80D/20E in 20 min. UV detection was performed at 280 nm, and all samples were injected in triplicate. Chromatographic data were processed and the fractions were pooled. (C), Lane M, molecular weight markers; and Lane1 cell lysates from *E. coli* strain BL21(DE3)/pET11a-MpChi-1, after 0.5 mM IPTG induction of protein expression and future cultivation for 3.5 h at 37°C; Lane2, cell lysates from *E. coli* strain BL21(DE3)/pET11a, after 0.5 mM IPTG induction of protein expression and future cultivation for 3.5 h at 37°C; Lane 3, purified MpChi-1 protein after ammonium sulphate precipitation (80% saturation) and preparative reversed phase HPLC on a Jupiter C18 column (250 × 4.6 mm, 5 μm particle size) using a water/acetonitrile buffer system.

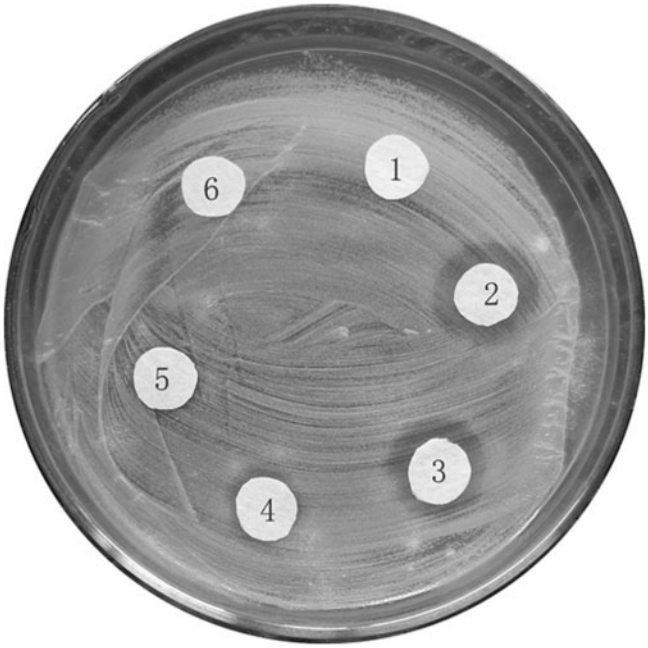


Fig. 5. **Inhibition the growth of *Gloeosporium musarum* by the purified recombinant chitinase.** Amounts of the purified recombinant chitinase applied were as follows: disk 1, 0 μg; disk 2, 40 μg; disk 3, 30 μg; disk 4, 20 μg; disk 5, 10 μg; disk 6, 5 μg.

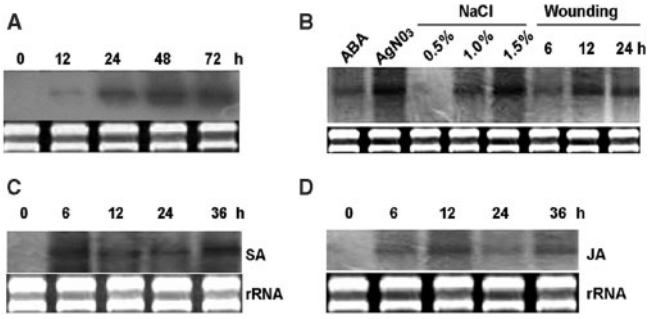


Fig. 6. **Induced expression of *MpChi-1* mRNA by various stimuli.** (A) Accumulation of *MpChi-1* mRNA in response to fungal infection; RNA isolation from plantain leaves was collected at the time points (h) indicated in the top of the figure after inoculating plantain leaves against *Gloeosporium musarum*. Total RNA (30 μg/lane) was separated in a formaldehyde/agarose gel, transferred onto a nylon membranes and hybridized with a [^{32}P]-labelled cDNA fragment; (B) northern blotting analysis accumulation of *MpChi-1* mRNA after wounding and exposure to osmotic stress (NaCl), ABA and AgNO3 solution;(C) Accumulation of *MpChi-1* mRNA in response to SA (2 mmol/l); (D) Accumulation of *MpChi-1* mRNA in response to JA (50 μmol/l).RNA isolation from plantain leaves was collected at the time points (h) indicated in the top of the figure after the SA or JA treatment. Total RNA (30 μg/lane) was separated in a formaldehyde/agarose gel, transferred onto a nylon membranes and hybridized with DIG- labelled *MpChi-1* cDNA fragment. Lower panels show the ethidium bromide staining of the RNA samples analysed.Equal amounts (30 μg) of RNA were loaded in each lane for hybridization analysis.

potential for crop protection by its heterologous expression. Numerous plant and microbial chitinase cDNAs have been cloned. Some of these have been introduced into plants under the control of constitutive promoters, resulting in an enhancement of the host plant to fungal pathogens (*33*). This suggests we could transform *MpChi-1* to the plant and enhance the disease resistance ability in transgenic plant.

Defence-related responses of plant cells depend on a complex network of signal transduction pathways. SA may be a key component in systemic acquired resistance (SAR) (*34*, *35*), and efficiently induce cluster of SAR marker proteins such as PRP. SA is probably the most well-characterized signalling factor in plant defence reactions and is known as a potent inducer of many chitinases during pathogenesis, but signal transduction pathways involving methyl JA and ethylene have also been characterized (*36*). Northern results indicated that SA could induce the gene transcript accumulation (Fig. 6C) suggesting that *MpChi-1* gene may be involved in SA-regulated defence responses. Although SA induced β-1,3-glucanase and chitinase in *Arabidopsis thaliana* (*37*), other researchers have reported conflicting results in tomato (*38*). Application of exogenous SA has long been known to stimulate expression of PR genes and to induce resistance to plant diseases (*39*, *40*). When applied to the leaves of TMV-resistant tobacco, SA was observed to induce the genes that were expressed relatively early (*41*). Our northern results showed that JA was also effective in inducing *MpChi-1* mRNA (Fig. 6D). These results indicated that JA may be a primary regulator of *MpChi*-1 expression during resistance to diseases.

To some extent, the *MpChi-1* was induced in leaves by wounding (Fig. 6B). Osmotic stress induced a slight accumulation of *MpChi-1* transcripts (Fig. 6B). These results suggested that the *MpChi-1* may be also preferentially involved in HR or biotic cell death during interaction between host and pathogen. This is not surprising, for although there are transmissible wounding signals, most wounding responses are also expressed in the wounded tissue or in the cells adjacent to the damaged cells (*42*). The speed and maintenance of the response seems to depend on the types of organs wounded, treatment procedures and which chitinase is examined. For example, peroxidase activity was detected at the wound site in tomato lateral endosperm after cutting (*43*) and plants attacked by pathogens rapidly deposit callose, a β-1,3-glucan, at wound sites (*44*).

ABA is a hormone that increases in concentration in the response of plants to a wide variety of abiotic stresses (*45*). To date, the majority of studies have characterized ABA as a negative regulator of plant defence responses, with only a few exceptions (*46*). In our case, ABA induced only a slight *MpChi-1* expression, if any (Fig. 6B). We propose that the *MpChi-1*, which encodes a chitinase with substantial anti-fungal characteristics, is a useful genetic tool to provide higher disease resistance for plants. Further, biochemical and molecular characterization of *MpChi-1*, including an evaluation of the regulatory elements imparting the observed induction patterns,

anti-fungal activities of the protein encoded by the gene, and demonstration of their usefulness in transgenic plants to enhance the disease resistance will broaden our knowledge of plant defence mechanisms.

It is generally assumed that the initiation of a plant's defence response depends on the recognition of molecular structures occurring in pathogens, or elicitors, by plasma membrane receptors (*47*, *48*). Perception of the pathogen, in turn, triggers the host response. Interestingly, our results indicate that the MpChi-1 has the high identity with rice endochitinase (XP_468715). Does it imply that the conservation of plant defence system against pathogen attack?

In conclusion, the data reported here indicate that plantain develop an active defence response, in which signal transduction pathways operate to prevent colonization by invading fungus. This is an adaptive response against potential assaults (biological and otherwise) coming from the environment. Revealing the natural defence mechanisms that infected plantain leaves develop against *G. musarum* is of interest not only because it will increase the general knowledge of plant defence mechanisms, but also because it could lead to more effective strategies for the protection of crop species.

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