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Molecular Cloning and Characterization of a Chitinase-Homologous Gene from *Mikania micrantha* Infected by *Cuscuta campestris*

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Abstract *Mmchi1*, a putative chitinase gene from *Mikania micrantha* infected by the parasitic plant *Cuscuta campestris*, was cloned and characterized. *Mmchi1* is predicted to encode a 35.42-kDa polypeptide with a isoelectric point of 5.69. The corresponding genomic sequence contains two introns (1,171 and 621 bp). Phylogenetic analysis showed that the predicted Mmchi1 protein is related to class I and class II chitinases (glycoside hydrolase family 19). Mmchi1 is likely a class II chitinase, as the protein sequence lacks a cysteine-rich hevein domain at its N terminus. Southern blot analysis suggests that sequences related to *Mmchi1* exist in the genome of *M. micrantha*. Expression of *Mmchi1* in different tissues was analyzed by semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR). *Mmchi1* was constitutively expressed in shoots, whereas only low transcript levels were detected in other tissues. Transcript levels of *Mmchi1* in shoots of *M. micrantha* infected by *C. campestris*, as analyzed by RT-PCR and real-time PCR, significantly increased after 2 days post infection but markedly decreased during the following days. Transcript levels of *Mmchi1* determined by semiquantitative RT-PCR varied in noninfected shoots exposed to various stress factors. Elevated levels of *Mmchi1* transcripts were accumulated in response to mechanical wounding and application of abscisic acid, salicylic acid, or $ZnSO_4$, respectively. Under salt stress conditions, *Mmchi1* transcripts accumulated at significant lower levels, however. Taken together, these data suggest that *Mmchi1* is a stress-related gene of *M. micrantha*, which is stimulated in response to *C. campestris* infection at early post-penetration stage but significantly suppressed during later infection stages.

Keywords Chitinase · *Cuscuta campestris* · Gene expression · Glycoside hydrolase family 19 · *Mikania micrantha*

Abbreviations

dpi	Days post infection
MW	Molecular weight
pI	Isoelectric point
ORF	Open reading frame
PCR	Polymerase chain reaction
PR	Pathogenesis-related
RACE	Rapid amplification of cDNA ends
RT-PCR	Reverse transcriptase-polymerase chain reaction
SSH	Suppression subtractive hybridization

Introduction

Plants activate defense reactions in response to pathogen infection and environmental stress factors. One of the most important defense mechanism is the accumulation of pathogenesis-related (PR) proteins. Plant chitinases (EC 3.2.2.14) are typical PR proteins, some of which have been shown to play a crucial role in defense against fungi containing chitin in their cell walls (Collinge et al. 1993;

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Neuhaus 1999). On the basis of primary structures and specific domains, plant chitinase genes have been grouped into various classes belonging to the glycoside hydrolase families 18 and 19 (Henrissat 1991; Neuhaus et al. 1996).

Most plant chitinases cleave chitin or chitin oligosaccharides, and many of them possess lysozyme activity. Certain plant chitinases cleave glucosamine-containing arabinogalactan proteins isolated from plant cell walls (van Hengel et al. 2001). Furthermore, various chitinases are able to cleave bacterial lipo-chitooligosaccharidic nodulation factors in the *Rhizobium*–legume symbiosis (Staehelin et al. 1994; Schultze et al. 1998). Interestingly, some plant chitinase homologues lack chitinolytic activity and represent seed storage proteins, lectins, or xylanase inhibitors (e.g., Durand et al. 2005; Perlick et al. 1996; Van Damme et al. 2007).

Many plant chitinases are involved in protection against pathogen invasions. It has been reported that chitinases inhibit growth of chitin-containing fungi, both in vitro (Schlumbaum et al. 1986; Mauch et al. 1988b) and in vivo (Broglie et al. 1991; Maximova et al. 2006; Robert et al. 2002; Xiao et al. 2007). Expression of plant chitinase genes is often up-regulated either upon pathogen attack or in response to a variety of abiotic stress factors (Bailey et al. 2005; Hong and Hwang 2002; Keulen et al. 2008; Khan and Shih 2004; Porat et al. 2001; Shinya et al. 2007; Wu and Bradford 2003). Furthermore, some chitinase genes may also possess developmental and physiological functions, including processes related to somatic embryogenesis in carrot, spruce, and *Arabidopsis* (de Jong et al. 1992, 1995; Dong and Dunstan 1997; Passarinho et al. 2001), maturation of pea pods (Mauch et al. 1988a, b), senescence of rapeseed leaves (Hanfrey et al. 1996), and ripening of grape berries (Robinson et al. 1997).

Mikania micrantha, one of the 100 worst invasive alien species in the world (Lowe et al. 2001), is a perennial vine belonging to the Asteraceae family. The species is native to Central and South America, where it is a weed of minor importance (Holm et al. 1977; Wirjahar 1976). In recent decades, *M. micrantha* has caused severe damage to many ecosystems in southern China (Zhang et al. 2004). A previous study showed that *Cuscuta campestris* (field dodder) can suppress growth of *M. micrantha* (Parker 1972). *C. campestris*, belonging to the Convolvulaceae family, is a root and leafless plant living as holoparasite on various host plants (Dawson et al. 1994). Recently, the effects of *C. campestris* on *M. micrantha* have been studied with respect to biomass, physiology, and ecology. For example, pot trials showed that *C. campestris* significantly reduced the total biomass, changed the biomass allocation patterns, completely inhibited the flowering, and also reduced photosynthesis and growth of the parasitized *M. micrantha* plants (Shen et al. 2005, 2007). Furthermore, field trials showed that *C. campestris* significantly reduced the biomass of *M. micrantha* (Lian et al. 2006). However, no information is available on the function of PR genes of *C. campestris*-infected *M. micrantha* plants.

In a previous study, we isolated a complementary DNA (cDNA) fragment, EY202328, from a suppression subtractive hybridization (SSH) cDNA library of *M. micrantha* shoots infected by *C. campestris* (Li et al. 2009). EY202328 shows sequence similarities with various plant chitinase genes. Here, we cloned a full-length *M. micrantha* chitinase gene (*Mmchi1*) using rapid amplification of cDNA ends (RACE). Bioinformatic analysis of the amino acid sequence deduced from *Mmchi1* cDNA predicts a putative class II chitinase belonging to glycoside hydrolase family 19. Expression profiles of *Mmchi1* in *M. micrantha* shoots infected by *C. campestris* were investigated at different time points using semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR) and real-time polymerase chain reaction (real-time PCR). Finally, levels of *Mmchi1* transcripts in noninfected shoots treated with different chemicals were determined by semiquantitative RT-PCR.

Materials and Methods

Plant Material, Growth Conditions, and Infection of *M. micrantha* with *C. campestris*

Whole *M. micrantha* H. B. K. (climbing hempweed) plants were collected from a population in Zhuhai, Guangdong province, China. Two-node segments, similar in size, were planted into pots (18 cm in height, 22 cm in upper diameter, and 15 cm in lower diameter), which were filled with a mixture of humus soil, perlite, and sand (3:1:1 in volume). Plants were maintained under greenhouse conditions for 2 months (25±1°C; 75±10% relative humidity; 300 μmol photons per square meter per second light intensity with a 16-h light–8-h dark photoperiod). A bamboo cane (about 1.5 m in length) was placed vertically in each pot, on which *M. micrantha* climbed. *Cuscuta campestris* Yuncker (field dodder) was cultivated on *Wedelia trilobata* (L.) Hitch. under the same growth conditions as for *M. micrantha* plants. *W. trilobata* was chosen as the "donor host" for *C. campestris* cultivation. *W. trilobata* displayed a high tolerance to *C. campestris* infection and remained alive for a long time. Healthy 2-month-old *M. micrantha* plants were used for experimental treatments and nucleic acid extraction.

For infection of *M. micrantha* plants, *C. campestris* shoots (about 20 cm in length) were coiled around the green stem (80 cm in height) of *M. micrantha*, and this time point was considered as the initial time of the infection process. Noninfected and infected plants were watered with sterile distilled water once a day and grown as described above.

Three *M. micrantha* shoots were excised at 0, 2, 4, 7, and 14 days postinfection (dpi), respectively. Collected samples were washed with distilled water, dried with filter paper, immediately frozen in liquid nitrogen, and stored at −80°C until extraction of total RNA.

RNA Extraction and SSH cDNA Library

Total RNA was extracted as previously described (Ding et al. 2008). Total RNA for SSH and RACE analyses was extracted from *M. micrantha* shoots at 2 dpi, while RNA for gene expression analysis was obtained from plants harvested at different time points. Total RNA (1.0 μg) from infected plants (2 dpi) and noninfected control plants was used to produce SMART cDNA using the SMART™ PCR cDNA Synthesis Kit according to the manufacturer's protocol (Clontech, Mountain View, USA). As reported previously (Li et al. 2009), a forward subtractive cDNA library was constructed using the PCR-select cDNA Subtraction Kit according to the manufacturer's instructions (Clontech, Mountain View, USA). RNA from shoots of infected *M. micrantha* was used as the "tester," while RNA from shoots of noninfected *M. micrantha* was used as the "driver." After two rounds of subtractive hybridization and two rounds of suppressive PCR, PCR products were ligated into the pGEM-T Easy vector (Promega, Madison, USA) to construct a *C. campestris*-infected SSH cDNA library for *M. micrantha*. To identify differentially expressed genes in *M. micrantha* during *C. campestris* infection, 250 white clones were randomly picked from the library and sequenced. Editing, BLAST alignments (Altschul et al. 1997), and functional annotation of these sequences were reported recently (Li et al. 2009).

Rapid Amplification of cDNA Ends

Through BLAST searches, a 714-bp cDNA of *M. micrantha* (accession number EY202328) was identified, which showed moderate identity with plant chitinase genes (Li et al. 2009). To obtain a full-length cDNA, a pair of gene-specific primers (3′F1: 5′-TGGTGCTTTGCCTGTCTTCT-3′; 5′R1: 5′-ATGGAGTGGCAACTGGAGGG-3′) was deduced from the EY202328 sequence and synthesized (Invitrogen Biotech, Shanghai, China; Fig. 1a). 5′ and 3′ RACE were performed using a SMART™ RACE cDNA Amplification Kit (Clontech, Mountain View, USA). The unsubtracted tester's single-strand cDNA (diluted 1×10^{-2}) was used as a template. The PCR reaction system (25 μl) contained 12.5 μl 2×GC buffer I, 4.0 μl dNTP (2.5 mM each), 0.25 μl *LA* Taq DNA polymerase (5U/μl; TaKaRa, Dalian, China), 1.0 μl of each gene-specific primer and adapter primer (10 μM each), 1.0 μl template DNA, and 5.25-μl distilled water. PCR reactions were performed in a PTC-200 thermocycler (MJ Research, USA) as follows: (1) an initial denaturation step at 94°C for 4 min, (2) 31 cycles of amplification [denaturation at 94°C for 30 s, annealing at 60°C for 30 s (5′ RACE) or at 56°C for 30 s (3′ RACE), extension at 72°C for 1 min], and (3) a final extension step at 72°C for 10 min.

By aligning the nucleotide sequences from EY202328 and the 5′ and 3′ RACEs, the entire coding region of *Mmchi1* cDNA was predicted and amplified with a pair of gene-specific primers (5′F2: 5′-ATGGAGACAAAAAG-TATG-3′; 3′R2: 5′-AAGCTTACTTGAATCCGAGTCT-3′; Fig. 1a). The unsubtracted tester's single-strand cDNA (diluted 1×10^{-2}) was used as a template. PCR reactions were carried out using (1) an initial denaturation step at 94°C for 4 min, (2) 31 cycles of amplification (94°C for 30 s, 58°C for 30 s, and 72°C for 1 min), and (3) a final extension at 72°C for 10 min (*LA* Taq polymerase, TaKaRa, Dalian, China).

PCR products were detected on 1.0% agarose gels stained with ethidium bromide, purified by the QIAquick gel extraction kit (Qiagen, Hilden, Germany) and cloned into the pGEM-T Easy vector (Promega, Madison, USA). The constructs were then transformed into competent cells of *Escherichia coli* strain DH5α (Dingguo, Beijing, China). White clones were checked by PCR, and positive clones were sequenced (Invitrogen Biotech, Shanghai, China).

Isolation of *Mmchi1* from *M. micrantha* Genomic DNA

Genomic DNA was extracted from shoots of 2-month-old *M. micrantha* plants as described previously (Li et al. 2007). The gene-specific primers (5′F2 and 3′R2) were used for amplification of *Mmchi1* gene from isolated genomic DNA. The PCR reaction system (25 μl) contained 12.5 μl 2×GC buffer I, 4.0 μl dNTP (2.5 mM each), 0.25 μl *LA* Taq DNA polymerase (5U/μl; TaKaRa, Dalian, China), 1.0 μl of each gene-specific primer (10 μM each), 1.0 μl genomic DNA (100 ng/μl), and 5.25-μl distilled water. Reactions were performed in a PTC-200 thermocycler (MJ Research) with a denaturation step at 94°C for 4 min, followed by 35 cycles (denaturation at 94°C for 30 s, annealing at 58°C for 30 s, extension at 72°C for 2.5 min), and a final extension step at 72°C for 10 min. PCR products were gel-purified, subcloned, and sequenced (Invitrogen Biotech). Finally, the obtained DNA sequence was analyzed using GENSCAN (Burge and Karlin 1997).

Bioinformatic Analysis

DNA sequence data were analyzed using BLAST (http://www.ncbi.nlm.nih.gov/blast; Altschul et al. 1997), CAP3 Sequence Assembly Program (http://pbil.univ-lyon1.fr/cap3.php), ORF Finder (http://www.ncbi.nlm.nih.gov/gorf.

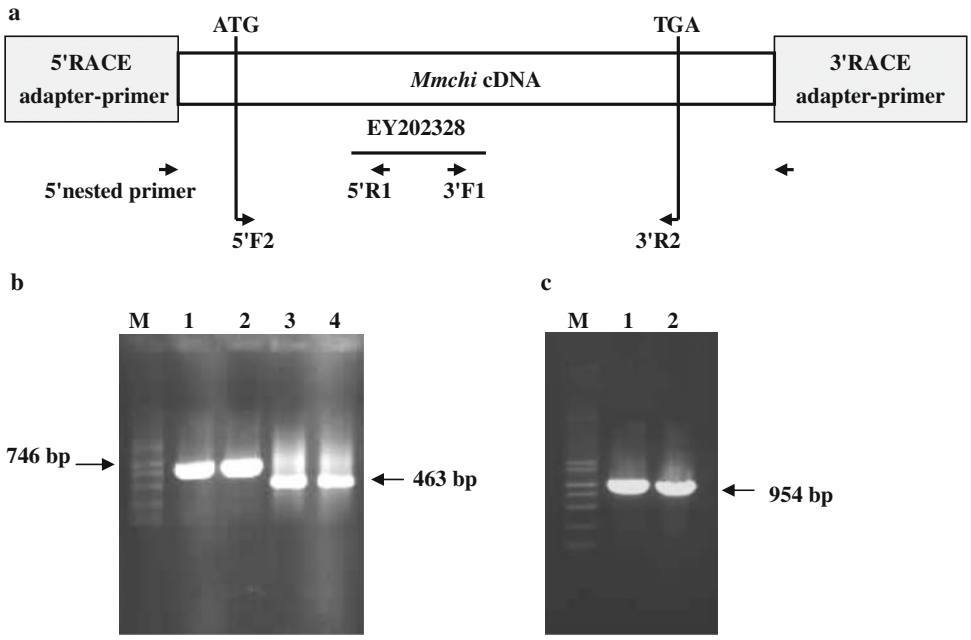

Fig. 1 **a** Graphical presentation of the cloning strategy used for isolation of a full-length *Mmchi1* cDNA. **b** Electrophoresis gels of amplified fragments of *Mmchi1* cDNA. A 746-bp fragment (*lanes 1* and *2*) was amplified through a 3′-RACE-PCR approach and a 463-bp fragment (*lanes 3* and *4*) was amplified through a 5′-RACE-PCR approach. **c** Amplification of a cDNA fragment of 954 bp (*lanes 1* and *2*), containing the entire coding region. *Lane M*, DGL 2000 DNA marker

html), InterProScan (http://www.ebi.ac.uk/InterProScan), TMHMM 2.0 (http://www.cbs.dtu.dk/services/TMHMM), NetNGlyc 1.0 (http://www.cbs.dtu.dk/services/NetNGlyc), TargetP 1.1 (http://www.cbs.dtu.dk/services/TargetP), SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP), and the Compute pI/Mw tool (http://www.expasy.ch/tools/pi_tool.html). Multiple alignments were carried out using the ClustalX software version 1.81 (Thompson et al. 1997). The phylogenetic tree was constructed by the neighbor-joining method (1,000 bootstrap replicates) using the MEGA program version 3.1 (Kumar et al. 2004).

Southern Blot Analysis

Aliquots of genomic DNA (20 μg) were digested at 37°C overnight with restriction endonucleases, *Bam*HI, *Eco*RI, and *Hin*dIII (TaKaRa, Dalian, China), separated by electrophoresis on an 0.8% (*w*/*v*) agarose gel (20 μg per lane), and transferred onto a Hybond-N^+ nylon membrane (Roche, Germany), according to manufacturer's instructions (Sambrook et al. 1989). The probe was obtained by using PCR with a pair of gene-specific primers (forward, 5′-GCACCACCCAGAATACCTT-3′, reverse, 5′-TTCTGAG TAAGGAGGCAAT-3′), which was based on the first intron sequence of *Mmchi1* gene. The resulting PCR product spanned 886 bp of the first intron of *Mmchi1*. The DNA was labeled with digoxigenin-11-dUTP by random priming using the DIG-High Prime DNA Labeling and Detection Kit (Roche Diagnostics, Mannheim, Germany). Hybridization was conducted according to the manufacturer's instructions.

Expression Analysis of *Mmchi1* in *M. micrantha* infected by *C. campestris*

Total RNA was isolated from *M. micrantha* shoots at 0, 2, 4, 7, and 14 dpi, respectively. Prior to cDNA synthesis, all RNA samples used for semiquantitative RT-PCR were treated with RNase-free DNase (TaKaRa) to remove DNA contamination. First-strand cDNA of each sample was synthesized by using the Prime-Script™ Reagent Kit (TaKaRa) according to the manufacturer's protocol. The PCR reaction system (25 μl) contained 2.0 μl of first-strand cDNA (10×diluted) and 0.2 μM (final concentration) of *Mmchi1*-specific primers (forward, 5′-GCACCACCCA GAATACCTT-3′; reverse, 5′-GGGACCCAGCATACT CACG-3′). The PCR was carried out as follows: (1) a denaturation step at 94°C for 3 min, (2) 35 amplification cycles (30 s at 94°C for denaturation, 30 s at 58°C for annealing, 45 s at 72°C for synthesis), and (3) a final extension step at 72°C for 5 min. The amplified *Mmchi1* fragment was 299 bp in length. The *M. micrantha* actin gene (*Mmactin*, accession number EY456955) was used as an internal control and amplified with specific primers (forward, 5′-CGGTCTTTCCCAGTATTGTA-3′; reverse, 5′-GTTT AGTGGTGCCTCGGTGA-3′). The PCR for *Mmactin* was similar to the PCR for *Mmchi1* but with only 30 amplification cycles. The amplified fragment of *Mmactin* was 248 bp in length. Seven microliters of each reaction mixture were analyzed on 1.0% (*w*/*v*) agarose gels (in TAE buffer).

Expression of *Mmchi1* in shoots of infected *M. micrantha* was further analyzed by real-time PCR using *Mmactin* gene as an internal standard. Each real-time PCR was conducted

in triplicate. The primer sequences for *Mmchi1* and *Mmactin* were as follows: *Mmchi1* forward, 5′-GCACCACCCAGAA TACCTT-3′, *Mmchi1* reverse, 5′-GGGACCCAGCATACT CACG-3′, *Mmactin* forward, 5′-AGGCGGGATTTGC TGGT-3′, and *Mmactin* reverse, 5′-TACCTCTTTTG GACTGGGCTTC-3′. Real-time PCR was carried out with the real-time SYBR green kit (TaKaRa) in a final volume of 25 μl, which contained 0.2 μM of each primer, 12.5 μl SYBR Premix (2×), 2 μl of first-strand cDNA (10×dilution), and distilled water. PCR reactions were performed in optical reaction strip tubes using an iCycler iQ5 apparatus (BioRad). The cycling conditions were as follows: (1) a preheating step for 1 min at 95°C followed by (2) 40 cycles (denaturation for 10 s at 95°C, annealing for 15 s at 59°C, extension for 15 s at 72°C, and data acquisition at 81°C). At the end of the amplification experiment, a melting curve was established (between 55°C and 95°C by steps of 0.5°C) to ensure that the signal corresponded to a single PCR product. Plasmids carrying DNA for each gene were used to prepare the standard curves, and gene quantity was deduced from the standard curves. Only amplifications of primer pairs with a single PCR product, and a determination coefficient (R^2) of the standard curve equal or superior to 0.99 were considered as valid. For quantification of *Mmchi1* transcript levels in a given tissue, the *Mmchi1* data were normalized to the average amount of *Mmactin* transcripts. Normalized values from three replicates were used to calculate the average relative expression ratios (*Mmchi1*/*Mmactin*).

Expression Patterns in Different Tissues and Stress Treatments

To estimate gene expression in different tissues, *M. micrantha* samples were collected from leaves, roots, stems, and shoots 2 months after two-node segments were planted into pots.

To investigate expression of *Mmchi1* gene in response to stress factors, 2-month-old noninfected *M. micrantha* plants (about 1.5 m in height) were treated with various chemical stimuli. *M. micrantha* plants were sprayed with a solution containing 20 mM $ZnSO_4$, 100 μM abscisic acid, 2 mM salicylic acid, or 500 mM NaCl. Mechanical wounding was performed by injuring shoots (8 cm in length) with needles. Fifteen *M. micrantha* plants were used for each treatment. Plants were kept in a growth room under the same conditions as described above. For each treatment, samples from three shoots were collected at 6, 24, 48, and 96 h and then immediately frozen in liquid nitrogen. The plant material was stored at −80°C until RNA extraction.

Expression patterns of *Mmchi1* in different tissues and in response to different stress treatments were analyzed by semiquantitative RT-PCR as described above.

Results

Cloning of a *Mmchi1* cDNA

PCR with primers deduced from a *M. micrantha* gene fragment (EY202328) followed by 3′ RACE and 5′ RACE resulted in three DNA fragments of around 750, 500, and 1,000 bp, as detected on 1% agarose gels (Fig. 1b, c). The fragments were cloned into the pGEM-T Easy vector and sequenced (746 bp for 3′ RACE, 463 bp for 5′ RACE, and 954 bp for the coding region fragment). CAP3 software was used to combine the fragments into a 1,295-bp cDNA sequence (called *Mmchi1*). BLAST search with the obtained *Mmchi1* nucleotide sequence indicated that it shared a high degree of sequence identity (74%) with *Gossypium hirsutum* chitinase-like protein (accession number AY291285) and certain sequence similarities with other plant chitinase genes (data not shown). The region from 52 to 1,008 nucleotides corresponds to an open reading frame encoding a polypeptide of 318 amino acids with a predicted molecular mass of 35.42 kDa and an isoelectric point of 5.69. The *Mmchi1* sequence is likely a full-length cDNA since it contained a predicted start codon region, AAAATGG, which is in agreement with the Kozak consensus initiator ANNATGG (Lutcke et al. 1987) as well as a polyadenylation signal (AATAA) in its 3′ untranslated region. The *Mmchi1* sequence has been submitted to the GenBank database (accession number EU716625).

Analysis of the Genomic *Mmchi1* Sequence

Primers deduced from the identified *Mmchi1* cDNA sequence were used to amplify the corresponding genomic DNA of *M. micrantha*. The amplified 2,749-bp DNA fragment of *Mmchi1* (from the start codon to the termination codon) has been submitted to the GenBank database (accession number FJ221367). The exon/intron boundaries of the genomic sequence were determined by GENSCAN analysis, and the genomic sequence was aligned with the corresponding cDNA sequence. The genomic DNA of *Mmchi1* contained two introns with a size of 1,171 and 621 bp, respectively (Fig. 2). The first intron had T/GT and AG/G, whereas the second intron had G/GT and AG/G splice site sequences. All sequences of the introns followed the "GU-AG rule" for *cis*-splicing (Blumenthal and Steward 1997). The size of *Mmchi1* exons ranged from 160 to 418 bp with an average exon size of 319 bp (Fig. 2).

Analysis of the Deduced Amino Acid Sequence of *Mmchi1*

A search at the European Bioinformatics Institute homepage for conserved protein domains indicated that the

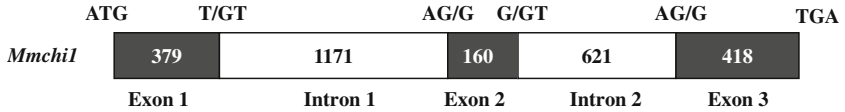

Fig. 2 Genomic structure of the *Mmchi1* gene. *Black boxes* indicate exons and *open boxes* represent introns. Exons and introns are drawn schematically to indicate their relative position and size

predicted amino acid sequence of *Mmchi1* contained a glycoside hydrolase family 19 (Pfam) domain (from position 65 to 282), a chitinase-related domain (from position 138 to 310) and a lysozyme-like superfamily domain (from position 56 to 309). NetNGlyc program analysis predicted five putative N-glycosylation sites, all of them with a probability greater than 50%. A signal peptide was predicted with the program SignalP, and the most likely cleavage site was between positions 23 and 24: VDA-DT. Prediction of transmembrane helices with TMHMM 2.0 suggests that Mmchi1 has a transmembrane helix domain, which is located at position 7–29. The TargetP program predicts that Mmchi1 is secreted via the secretory pathway.

A protein BLAST sequence similarity search with the Mmchi1 sequence indicated that Mmchi1 shared high sequence identity with genes encoding class I, class II, and class VII chitinases of diverse plant species including class I chitinases of *Gossypium barbadense* (accession number ABL86683, 75% identity), *Pisum sativum* (BAC81645, 70% identity), *Limonium bicolor* (ABD92820, 66% identity), and *Arabidopsis thaliana* (NP_172076, 65% identity), class II chitinases of *G. barbadense* (ABL86684, 74% identity), *Pyrus pyrifolia* (ACM45714, 68% identity), and *L. bicolor* (ACE79211, 66% identity), and a class VII chitinase of *G. hirsutum* (AAP80800, 67% identity). Moreover, Mmchi1 exhibited sequence similarities with various other plant chitinases (sequence identity from 30% to 52%). Figure 3 shows a protein sequence alignment of Mmchi1 with related full-length sequences of class II chitinase proteins from *P. pyrifolia* (ACM45714), *L. bicolor* (ACE79211), and *A. thaliana* (NM_100466), respectively. To determine the phylogenetic relationship of Mmchi1 with chitinases from other plant species, the sequences of various chitinases from over 20 plant species (including representatives of the Asteraceae family and a class III chitinase from *Helianthus annuus*) were aligned, and a phylogenetic tree was constructed by using the neighbor-joining method. As shown in Fig. 4, phylogenetic analysis clearly separated the *H. annuus* class III chitinase sequence (glycoside hydrolase family 18) from the other chitinases belonging to glycoside hydrolase family 19. The Mmchi1 sequence of *M. micrantha* (ACD93719), *L. bicolor* (ACE79211), *P. sativum* (BAC81645), *P. pyrifolia* (ACM45714), *Ricinus communis* (EEF46716), *Medicago truncatula* (ABN08775), *A. thaliana* (NM_100466, NM_112568), *Brassica rapa* (ABV89660), *G. hirsutum* (AAQ56598, AAP80800), and *G. barbadense* (ABL86683) clustered into the same subgroup of glycoside hydrolase family 19. The obtained dendrogram revealed that Mmchi1 is closely related to class I and class II chitinase proteins belonging to the Plumbaginaceae (*Limonium*), Brassicaceae (*Arabidopsis*, *Brassica*), Rosaceae (*Pyrus*), Fabaceae (*P. sativum*; *Medicago*), Malvaceae (*Gossypium*), and Euphorbiaceae (*Ricinus*).

Southern Blot Analysis

To test whether *M. micrantha* possesses DNA sequences related to *Mmchi1*, genomic DNA gel blot hybridization analysis was performed with 886-bp fragment of the first intron as a probe. The results showed that the probe hybridized to three major genomic DNA fragments of genomic DNA digested with either *Bam*HI, *Eco*RI, or *Hin*dIII (Fig. 5). As the probe lacked corresponding restriction enzyme cleavage sites, it is suggested that *Mmchi1* is present in several copies in the *M. micrantha* genome or that *M. micrantha* possesses additional chitinase sequences closely related to *Mmchi1*.

Expression of *Mmchi1* Gene in Response to *C. campestris* Infection

Semiquantitative RT-PCR was performed to determine the expression profile of *Mmchi1* in *M. micrantha* plants harvested at different time points after *C. campestris* infection. Figure 6a shows the obtained results for *Mmchi1* and *Mmactin* transcript levels in shoots of infected *M. micrantha* plants. Elevated amounts of *Mmchi1* transcripts were measured at early stages of infection. Maximal expression levels were observed at 2 dpi, whereas accumulated *Mmchi1* transcripts decreased remarkably at later time points (from 2 to 14 dpi). Transcript levels of *Mmactin* from all samples were almost identical during the time course experiment.

Real-time PCR was performed to confirm the reliability of semiquantitative RT-PCR analysis and to accurately quantify the amount of *Mmchi1* transcripts in the different samples. The expression profile of *Mmchi1* in *M. micrantha* shoots infected by *C. campestris* at different time points is shown in Fig. 6b. The presented data reflect the amount of *Mmchi1* transcripts normalized to *Mmactin* at different sampling points. Highest levels of *Mmchi1* transcripts were

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At-NM_100466      MVTIRSGS-IVILVLLAVSFLAL-VANG-EDKTIK--VKKVRGNKVCTQGWECSWWSKYC 55
Pp-ACM45714       ---MEKKW-LLLFSMAAATLLAVDVANGQEESAVKPLVKIVKGKKVCDKGWECKGWSVYC 56
Mm-ACD93719       ---METKS-MCLLVLSVLFVMVS-IVDA-DTSPVL--VKKIKGKRVCDQGWECKGWSEYC 52
Lb-ACE79211       ---MGRHWRLVIVAIVTTALLGANLSAALDLTDQL-VTKEVKGKKVCKRGWACPEFSKFC 56
                     :     : :. : .  .:   :  . : .     .* ::*::** :** *  :* :*

At-NM_100466      CNQTISDYFQVYQFEQLFSKRNTPIAHAVGFWDYQSFITAAALFEPLGFGTTGGKLMGQK 115
Pp-ACM45714       CNQTISDYFQAYQFEDLFSKRNSPVAHAVGFWDYHSFITAAAEYQPHGFGTTGGKLQGMK 116
Mm-ACD93719       CNLTISQYFDYYQFENLFSKRNTPVAHAVGFWDFKSFITASAIYQPLGFGTTGNKTTQML 112
Lb-ACE79211       CNQTIPEFLDVDQFQQLFPNINAPTAHAVGFWDYQSFVLATIKYAPLGFGTTGGKLMGQL 116
                  ** **.::::  **::**.: *:* ********::**: *:  : * ******.*

At-NM_100466      EMAAFLGHVASKTSCGYGVATGGPLAWGLCYNREMSPMQSYCDESWKFKYPCSPGAEYYG 175
Pp-ACM45714       EVTAFLGHVGSKTSCGYGVATGGPTAWGLCYNKEMSPSQLYCDDYYKYTYPCSPGASYHG 176
Mm-ACD93719       EVAAFLAHVGSQTSCGYGVATGGPTAWGLCYNKEMSPMQDYCDDNYKYTYPCAPGAVYFG 172
Lb-ACE79211       ELAAFLGHVGSQTSCGYGVATGGPTAWGLCYNKELSPSQKYCDDSYKYTYPCTPGADYYG 176
                  *:::***.**.*:************* *******:*:** * ***: :*:.***:*** *.*

At-NM_100466      RGALPIYWNFNYGAAGEALKADLLNHPEYIEQNATLAFQAAIWRWMTPIKRAQPSAHDIF 235
Pp-ACM45714       RGALPLYWNYNYGETGDALKVDLLNHPEYIEQNATLAFQAAIWRWMTPVKKNIPSAHDVF 236
Mm-ACD93719       RGALPVFWNYNYGYIGDCIKADLLHHPEYLEQNATLAFQAAIFQWITPLKKGLPSAHDCM 232
Lb-ACE79211       RGALPIYWNYNYGQIGDALQENLLDHPEYIEQNATLAFQAAIYSWMTPKKKGQPSCHDAF 236
                  *****::**:***  *:.:: :**.****:*********: *:** *:  **.** :

At-NM_100466      VGNWKPTKNDTLSKRGPTFGSTMNVLYGEYTCGQGSIDPMNNIISHYLYFLDLMGIGRED 295
Pp-ACM45714       VGKWKPTKNDTLSKRVPGFGTTINVLYGDQVCGQGDVDSMNNIVSHYLYYLDKIGVGREE 296
Mm-ACD93719       VGSFKPTKNDTLSNRVPGFGCTMNILYGERTCGKGDIDDMNTIITHYLYYLDLMGFGREY 292
Lb-ACE79211       VGNWKPNKNDTLSKRVPGFGVTMNILYGDRTCGQGDVDDMNTIITHYLYYLDEMGVGRER 296
                  **.:**.******:* * ** *:*:***: .**:*.:* **.*::****:** :*.***

At-NM_100466      AGPNDELSCAEQKPFNPSTVPSSSSS 321
Pp-ACM45714       AGPHDVLSCAEQKAFQPSSSSSSSSS 322
Mm-ACD93719       AGSPEVLTCAEQKPFNPSTTKDSDSS 318
Lb-ACE79211       AGPAEVLSCAEQKPFNPSGSASS--- 319
                  **.  : *:*****.*:**   .*
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Fig. 3 Comparison of the predicted amino acid sequence of Mmchi1 with full-length sequences encoded by class II chitinase genes. The *numbers* on the right side indicate the amino acid positions in the sequence. The earmarks *dashes*, *asterisks*, *colons*, and *dots* indicate gaps, identical amino acid residues, conserved substitutions, and semiconserved substitutions in the aligned sequences. The sequences are indicated with the species name (*At*, *Arabidopsis thaliana*; *Lb*, *Limonium bicolor*; *Pp*, *Pyrus pyrifolia*; and *Mm*, *Mikania micrantha*) followed by the GenBank accession number

measured with *C. campestris*-infected plants harvested at 2 dpi. From 2 to 14 dpi, the accumulation of *Mmchi1* transcripts decreased significantly from 4 to 14 dpi. Hence, the semiquantitative RT-PCR and real-time PCR results showed a consistent expression profile. These data indicate that, after a transient stimulation at 2 dpi, accumulation of *Mmchi1* transcripts are reduced in *C. campestris*-infected *M. micrantha* shoots.

Expression of *Mmchi1* in Different Tissues

Accumulation of *Mmchi1* transcripts in different tissues of noninfected *M. micrantha* was examined using semiquantitative RT-PCR analysis. As shown in Fig. 7a, *Mmchi1* was constitutively expressed in shoots. In stem and root tissues, *Mmchi1* transcripts accumulated at relatively low levels. Transcripts of *Mmchi1* in leaves and petioles were barely

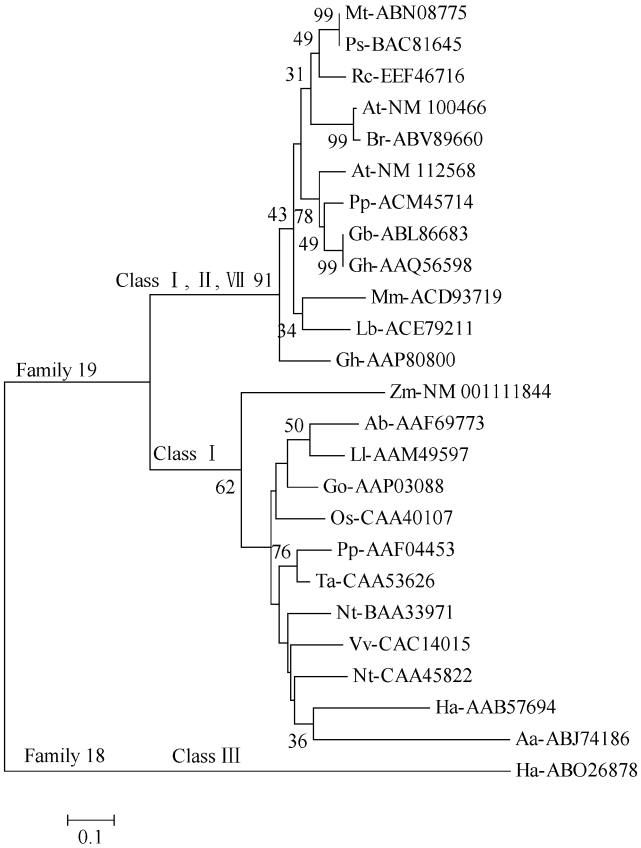

Fig. 4 Phylogenetic relationship between Mmchi1 and other chitinase proteins from various plant species. Branch lengths are proportional to the number of substitutions per site (the *scale bar* represents a genetic distance of 0.1; *numbers* on the *tree* indicate bootstrap values). Proteins of the tree are indicated by species names (*Ab*, *Arabis blepharophylla*; *At*, *Arabidopsis thaliana*; *Aa*, *Artemisia annua*; *Br*, *Brassica rapa*; *Gh*, *Gossypium hirsutum*; *Gb*, *Gossypium barbadense*; *Go*, *Galega orientalis*; *Ha*, *Helianthus annuus*; *Lb*, *Limonium bicolor*; *Ll*, *Leucaena leucocephala*; *Mm*, *Mikania micrantha*; *Mt*, *Medicago truncatula*; *Nt*, *Nicotiana tabacum*; *Os*, *Oryza sativa*; *Ps*, *Pisum sativum*; *Pp*, *Poa pratensis*; *Pp*, *Pyrus pyrifolia*; *Rc*, *Ricinus communis*; *Ta*, *Triticum aestivum*; *Vv*, *Vitis vinifera*; and *Zm*, *Zea mays*) followed by the GenBank accession number

detected. Similar expression patterns were obtained from two additional independent experiments (not shown).

Expression of *Mmchi1* Gene in Response to Wounding and Chemical Stimuli

Semiquantitative RT-PCR was performed to elucidate expression profiles of *Mmchi1* in *M. micrantha* shoots under various stress conditions, namely mechanical wounding and exogenous application of $ZnSO_4$, abscisic acid, salicylic acid, and NaCl. Plants exposed to salt stress withered, whereas the treatments did not induce visible stress symptoms of *M. micrantha*. Upon mechanical wounding, relatively high levels of *Mmchi1* transcripts were detected after 6 h, and accumulation of transcripts remained high during the following days, although a slight decline was observed 48 h after the stress treatment (Fig. 7b). In the case of zinc stress (20 mM $ZnSO_4$), the levels of transcripts did not change at 6 h but were strongly elevated at 24 h after the application of $ZnSO_4$. Levels of transcripts were gradually reduced at later time points (Fig. 7c). Abscisic acid (100 μM) appeared to cause a slight increase of accumulated *Mmchi1* transcripts 6 h post application. Transcript levels seem to be reduced at 48 h and elevated again at 96 h (Fig. 7d). A treatment with salicylic acid (2.0 mM) resulted in a significant accumulation of *Mmchi1* transcripts. Levels were increased at 6 h after treatment, remained high at 24 h, and then decreased (Fig. 7e). In response to salt stress (500 mM NaCl), levels of *Mmchi1* transcripts were gradually reduced from 6 to 96 h after the treatment (Fig. 7f). Similar results were obtained from two additional independent experiments (not shown).

Discussion

In a previous study, we identified a chitinase gene fragment (accession number EY202328) from a SSH library of *M. micrantha* shoots infected by *C. campestris*. In the present

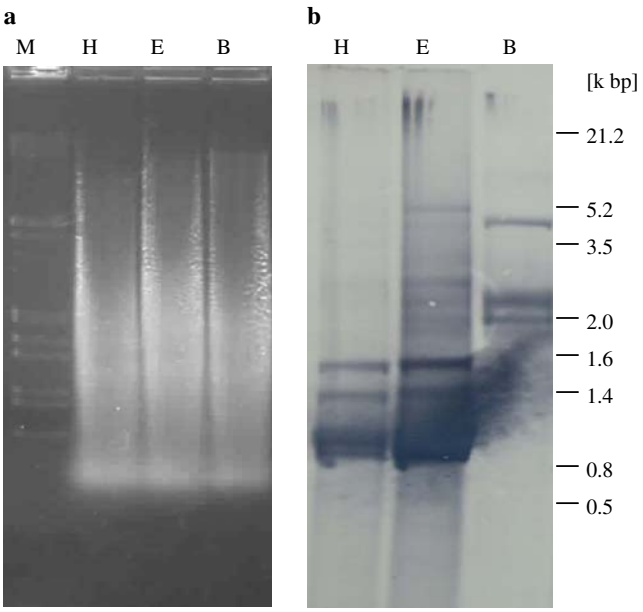

Fig. 5 Southern blot analysis of the *Mmchi1* gene. Genomic DNA of *M. micrantha* digested with the indicated restriction endonucleases was hybridized with the first intron of *Mmchi1* (886-bp fragment) as a probe. **a** Genomic DNA (isolated from *M. micrantha* shoots) was digested with *Bam*HI (lane B), *Eco*RI (lane E), and *Hin*dIII (lane H), separated on a 0.8% agarose gel by electrophoresis and visualized by ethidium bromide staining. *Lane M*, indicates molecular marker DNA (λDNA digested by *Eco*RI and *Hin*dIII). **b** Southern hybridization of the genomic DNA with the DIG-labeled 886-bp probe. Each lane contained 20 μg of the genomic DNA digested by *Bam*HI (lane B), *Eco*RI (lane E), and *Hin*dIII (lane H), respectively

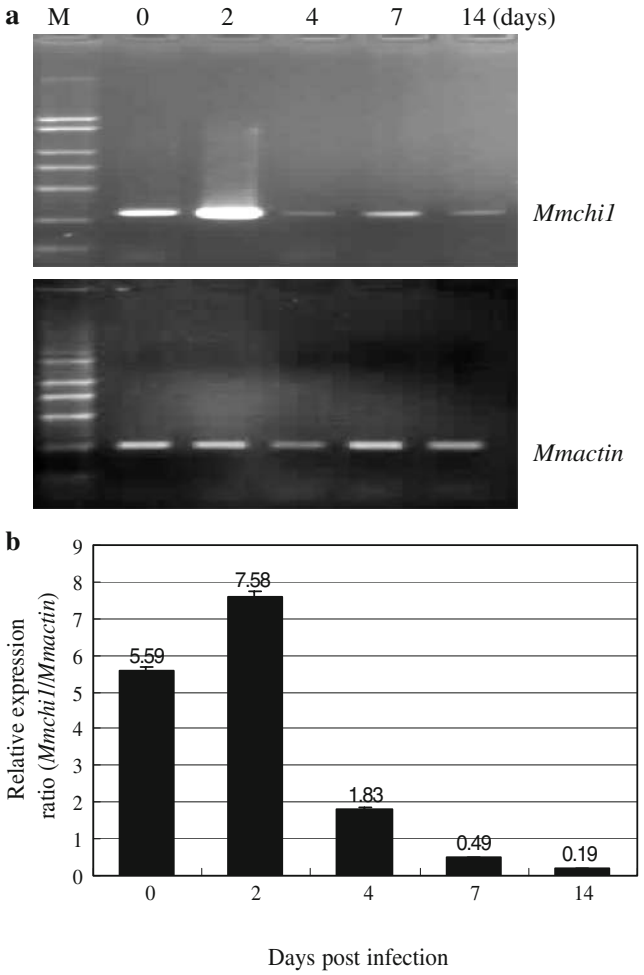

Fig. 6 RT-PCR and real-time PCR analysis of *Mmchi1* in *M. micrantha* shoots during *C. campestris* infection. **a** RT-PCR analysis of *Mmchi1* in infected *M. micrantha* shoots at different time points (dpi). **b** Relative real-time PCR analysis of *Mmchi1* in infected *M. micrantha* shoots at different time points (dpi). Data indicate *Mmchi1*/*Mmactin* ratios (amount of *Mmchi1* transcripts normalized to the transcripts of the *Mmactin* gene). *Error bars* represent the standard variation from three independent samples

study, we cloned and characterized the corresponding full-length chitinase gene, which was named *Mmchi1*. Similar to other chitinase genes, *Mmchi1* contained introns. Although intron sequences vary in different chitinases, the location of these introns seems to be conserved. Introns in *Mmchi1* were 1,171 and 621 bp in length (Fig. 2), whereas introns in other chitinases, such as strawberry *Fachi2-2* (Khan and Shih 2004), were considerably shorter. Phylogenetic analysis showed that the Mmchi1 protein is closely related to class I and class II chitinase proteins of species belonging to the Plumbaginaceae, Brassicaceae, Rosaceae, Fabaceae, Malvaceae, and Euphorbiaceae families. The Mmchi1 protein sequence lacks an N-terminal cysteine-rich domain (also called "hevein domain" or "chitin-binding domain"), which is present in all class I chitinases (Collinge et al. 1993; Neuhaus et al. 1996). Thus, we concluded that Mmchi1 is a putative class II chitinase (belonging to glycoside hydrolase family 19).

Three major hybridization bands were observed by Southern blot analysis with genomic DNA of *M. micrantha*, indicating that sequences closely related to *Mmchi1* exist in the *M. micrantha* genome. This observation is reminiscent to DNA hybridization results for chitinase genes from various other plant species (Nairn et al. 1997; Khan and Shih 2004; Porat et al. 2001; Robert et al. 2002; Xiao et al. 2007), suggesting that *M. micrantha* chitinase genes frequently duplicated during evolution.

Transcripts of *Mmchi1* differently accumulated in different tissues (Fig. 7a). Constitutive expression of *Mmchi1* was mainly detected in shoots, suggesting a specific role of *Mmchi1* in shoots. Previous studies revealed that various chitinase genes are developmentally regulated, suggesting a role in specific physiological processes (e.g., de Jong et al. 1992, 1995; Dong and Dunstan 1997; Hanfrey et al. 1996; Mauch et al. 1988a, b; Passarinho et al. 2001; Robinson et al. 1997; Staehelin et al. 1994). In sweet orange for example, transcripts of an acidic chitinase gene were expressed in young green bark but not in leaves, roots, or flavedo (Nairn et al. 1997). In balsam pear, transcripts of a class I chitinase gene (*Mcchit1*) were detected in unwounded leaves, roots, stems, and fruits by Northern blot analysis (Xiao et al. 2007).

Two different methods (semiquantitative RT-PCR and real-time PCR) were used to characterize the expression profile of *Mmchi1* gene during *C. campestris* infection. The data obtained with the two detection methods were consistent. Maximal accumulation of *Mmchi1* transcripts was observed as early as 2 dpi. This is reminiscent to the expression of two PR genes (*chitinase* and *defensin*), which were induced in resistant sunflower plants during *Orobanche cumana* infection at early (2–8 h) and later (13–21 days) stages of infection (Letousey et al. 2007). It is worth mentioning in this context that *C. campestris* infection inhibited growth of *M. micrantha* at 20 dpi and later time points (Shen et al. 2005, 2007). It is tempting to speculate that *Mmchi1* expression is rapidly induced by *C. campestris* infection at early post-penetration stages and that the successful invasion of *C. campestris* induces molecular mechanisms that suppress expression of PR genes in the host plant *M. micrantha.*

Previous studies reported that chitinase gene expression is modified by various treatments, such as wounding, application of plant hormones, hot water, and various stress-inducing chemicals (Bailey et al. 2005; Keulen et al. 2008; Porat et al. 2001; Shinya et al. 2007; Wu and Bradford 2003; Xiao et al. 2007). In this study, levels of accumulated *Mmchi1* transcripts were modulated by mechanical wounding, $ZnSO_4$, abscisic acid, salicylic acid,

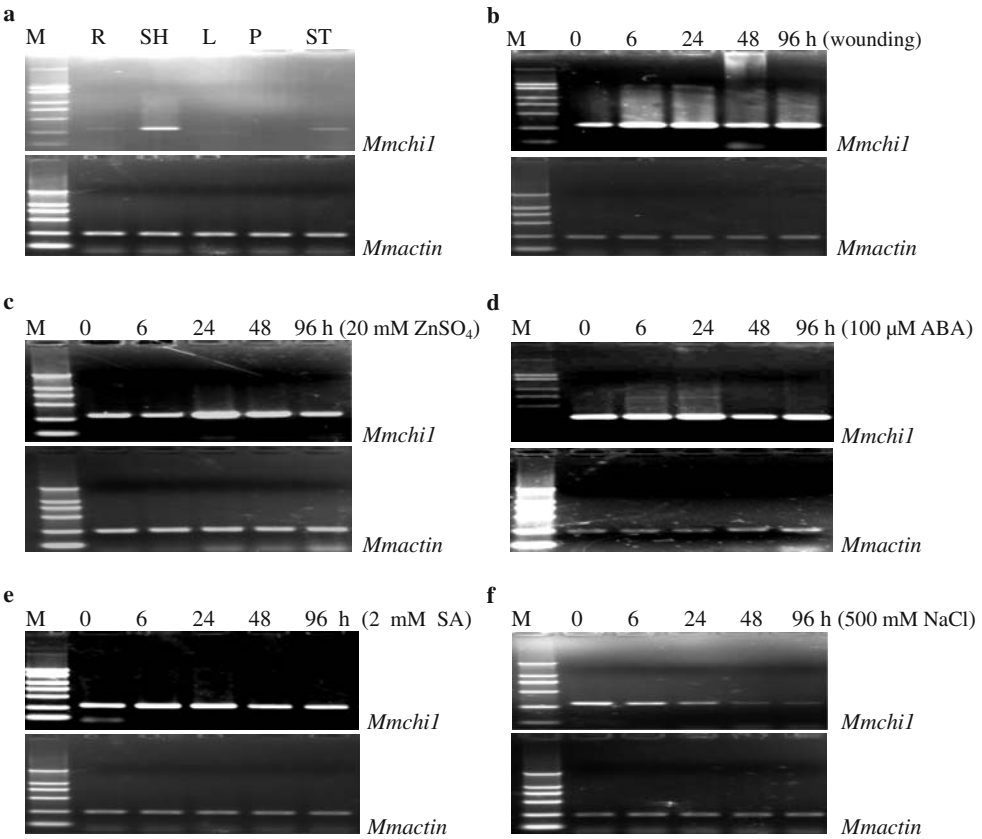

Fig. 7 Semiquantitative RT-PCR analysis of *Mmchi1* in noninfected *M. micrantha* plants. *Mmactin* was used as an internal control. **a** Expression pattern of *Mmchi1* in different tissues. Total RNA was extracted from leaves (L), roots (R), stems (ST), shoots (SH), and petioles (P) from 2-month-old *M. micrantha* plants. **b**–**f** Expression of *Mmchi1* in shoots of *M. micrantha* in response to mechanical wounding, 20 mM $ZnSO_4$, 100 µM abscisic acid (ABA), 2 mM salicylic acid (SA), and 500 mM NaCl. Plants were harvested at the indicated time points (hours after the treatment)

and high salt stress (Fig. 7b–f). The induction of *Mmchi1* by mechanical wounding points to a role of *Mmchi1* in plant defense, as physical damage of plant tissues provides potential infection sites for pathogens. In fact, the induction of various PR genes by wounding has been demonstrated in many studies (e.g., Bailey et al. 2005; Porat et al. 2001; Wu and Bradford 2003; Xiao et al. 2007). For example, a *chitinase* of *Theobroma cacao* (*TcChiB*) was induced by wounding in young red leaves but not in mature green leaves (Bailey et al. 2005). In our work, induction of *Mmchi1* in shoots was observed as early as 6 h after wounding, and maximal levels of transcripts were seen 24 h post treatment (Fig. 7b). This induction pattern is different from chitinase expression in grapefruit plants. Transcripts of *chi1* in grapefruit flavedo showed elevated levels after 24 h and reached a maximum 5 days after wounding (Porat et al. 2001).

In the present study, we found that abscisic acid and salicylic acid affected *Mmchi1* transcript levels in *M. micrantha* shoots. Exogenous application of the plant hormone abscisic acid resulted in an early and significant increase of accumulated *Mmchi1* transcripts (Fig. 7d). In contrast, abscisic acid had no effect on *Chi9* expression in tomato seeds and leaves (Wu and Bradford 2003). Salicylic acid has been demonstrated to play an important role in regulation of defense responses. In our study, exogenously applied salicylic acid slightly stimulated levels of *Mmchi1* transcripts within 6 h (Fig. 7e). In contrast, application of salicylic acid has been reported to suppress expression of *NtChitIV*, a tobacco chitinase gene induced in response to treatment of plants with a glucan elicitor from *Alternaria alternata* (Shinya et al. 2007).

The effect of the heavy metal Zn on accumulation of *Mmchi1* transcripts was also examined in this study. Our findings showed that Zn stress markedly increased levels *Mmchi1* transcripts 24 h after treatment with $ZnSO_4$ (Fig. 7c). This finding is similar to data from studies reporting on stress effects induced by various heavy metals. For example, transcript levels of a class III chitinase in leaves of dwarf sunflower (*H. annuus*) were strongly induced in response to As (Keulen et al. 2008).

In general, salt stress causes a serious cellular stress in most plants. High NaCl concentrations induce an osmotic stress that inhibits many cellular processes (Parida and Das 2005). In this study, the accumulation of *Mmchi1* transcripts was suppressed at high salt concentrations (Fig. 7f). As *M. micrantha* treated with NaCl withered, it is possible that effects of NaCl on *Mmchi1* expression are nonspecific and rather reflect disruption of cellular homeostasis induced by the stress treatment.

In conclusion, we have cloned and characterized a full-length chitinase gene from *M. micrantha*. Our data indicate that *Mmchi1* is constitutively expressed in various tissues and that transcripts mainly accumulated in shoots. Upon

infection by *C. campestris*, levels of *Mmchi1* transcripts were enhanced at the early post-penetration stage, whereas significantly lower levels were measured at later infection stages. Increased accumulation of *Mmchi1* transcripts was observed in response to mechanical wounding and $ZnSO_4$. Furthermore, our results point to the possibility that signal transduction pathways related to abscisic acid and salicylic acid modulate expression of *Mmchi1*. Taken together, these data suggest that elevated levels of *Mmchi1* transcripts reflect a stress response. Functional characterization of *Mmchi1* and other *M. micrantha* genes activated by *C. campestris* infection is in progress. Future experiments are required to understand the molecular mechanisms of induction and downregulation of stress-related genes in the *M. micrantha–C. campestris* interaction at various infection stages.

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