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Full Length Research Paper

Characterization and expression of *β -1, 3 glucanase* gene cloned from *Malus hupehensis*

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***MhGlu*, a *β*-1, 3-glucanase cDNA, was cloned from *Malus hupehensis* (Pamp.) Rehd by *in silico* cloning and validated with RT-PCR. *MhGlu* has an intron and possess 84, 83, and 77% nucleotide identity and 84, 74, and 76% amino acid identity with *Prunus persica*, *Prunus avium*, and *Vitis riparia*, respectively. *MhGlu* genomic DNA sequence and promoter sequence including the salicylic acid (SA) motif, methyl jasmonate (MeJA) responsive, and ethylene (ET) responsive elements were isolated. *MhGlu* expression was detected in *M. hupehensis* seedlings treated with SA, MeJA and 1-aminocyclopropane-1-carboxylic acid (ACC). Real-time quantitative RT-PCR (qRT-PCR) revealed constitutive expression of *MhGlu* in leaf but not in the stem and root where it was silent and induced by SA, MeJA, and ET. This result suggests that *MhGlu* might be involved in the SA- and the JA/ET-signaling pathways in *M. hupehensis*. The expression of the gene monitored in a 96 h course after inoculation with apple ring spot pathogen (*Botryosphaeria berengeriana* de Not. f. sp. *Parabola* Nise Koganezawa et Sakuma). Inoculation with *B. berengeriana*, up-regulated *MhGlu* 24 h post inoculation (PI), the expression reached to maximum at 48 h PI, and then decline. Moreover, apple aphid (*Aphis citricota* van der Goot) could enhance *MhGlu* expression in the leaf and stem compared to healthy control plants. It can be concluded from the results that *MhGlu* is involved in resistance to biotic stress in *M. hupehensis*.**

Key words: *β*-1, 3-glucanase, expression analysis, *Malus hupehensis,* promoter, salicylic acid (SA), jasmonic acid (JA), pathways.

INTRODUCTION

Plants are continuously attacked by pathogens and induce various defense responses to protect themselves (Chester, 1933; Boller, 1985; Van Loon, 2000). Systemic acquired resistance (SAR) is one of the most studied induced plant defense responses that are efficient against a broad spectrum of pathogens (Ryals et al., 1996). Salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) have emerged as key regulators activating the induced defense response. The *non-expresser of pathogenesis related genes*1 (*NPR*1) plays a key role in regulating SA mediated SAR. Upon treatment with SA, the NPR1 oligomer in the cytoplasm is reduced to active monomers which are transported to the nucleus. In the nucleus, they interact with the TGA family members of basic domain／leucine zipper (bZIP) transcription factors to induce the expression of *PR* genes which are the downstream molecules of SAR (Mou et al., 2003; Tada et al., 2008). ET could enhance SA/NPR1 dependent defense responses, modulates the role of *NPR1* in cross talk between SA and JA (Leon-Reyes et al., 2009). Plants are often invaded by multiple pathogens. Cross talk between induced defense signaling pathways may provide effective regulatory mechanisms for plant protection (Spoel and Dong, 2008).

Pathogenesis related (*PR*) genes, which are very

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important components induced by SAR pathway, have been studied in many plants, especially in model species to discover plant defense reaction (Tornero et al., 1997; Durrant and Dong, 2004). Based on their biochemical functions and amino acid (aa) sequences, PR proteins have been classified into 14 groups (PR-1 through PR-14) (Muthukrishnan et al., 2001). Classified in PR-2 family, *β*-1, 3-glucanase can protect plants against pathogen infection through two ways: one was directly weakening and decomposing fungal cell walls(Bartnicki-Garcia, 1968); and the other was indirectly releasing elicitors that can induce a chain of the consequent defense reactions through limited hydrolysis of the fungal cell wall (Lawrence et al., 2000) . The cloning and expression of *β-1, 3-glucanase* has been studied in herbaceous plants such as tobacco (Linthorst et al., 1990; Ernst et al., 1992), wheat (Liu et al., 2009; Liu et al., 2010), barley (Zeng et al., 2003), and plantain (Jin et al., 2007). Wheat *β-1, 3-glucanase* gene (*TaGluD*) transcript induction was more than 60-fold higher in the resistant Shannong0431 than in the susceptible Wenmai6 line after infection with *Rhizoctonia cerealis*, suggesting that *TaGluD* may be helpful for enhancing fungal resistance in several crop species (Liu et al., 2009). *FaBG2*-2 and *FaBG2*-3 expressions in the leaf of infected strawberry plants were induced by *Colletotrichum fragariae* and *Colletotrichum acutatum* (Shi et al., 2006). Highest expression was detected in strawberry leaf followed by fruit and relatively low expression recorded in the crown and root for both the genes (shi et al., 2006).

To our knowledge, few studies have investigated the role of *β-1, 3-glucanase* in woody plants, especially in fruit trees. *Malus hupehensis* apple rootstock, which has shown strong resistance to various apple pathogens, originated first in China and is important material to study woody plant resistance mechanisms (Lu et al., 1999). In this study, we isolated a *β-1, 3-glucanase* from *M. hupehensis* leaf treated by SA and examined expression of this gene in response to biotic and abiotic factors. This study provided us important information for understanding the molecular bases of *M. hupehensis β-1, 3-glucanase* and for study of the role of this gene in the defence of apple ring spot pathogen and apple aphid.

MATERIALS AND METHODS

BLAST-based searches and *in silico* cloning

The NCBI BLASTn program (http:// www.ncbi.nlm.nih.gov/blast) was used to search for the *β-1, 3-glucanase* gene in GenBank, nr, and expression sequence tag (EST) databases. The NCBI partial sequence of *β-1, 3-glucanase* (accession number EB156450) was used as an initial query sequence for the *Malus* × *domestic* ESTs. Additional iterative BLAST searches identified other related sequences to search for EST fragments for gene assembly. EST fragments (accessions number EB129003, FE969419, AM600693, and DR992840) were identified from the EST database and assembled using DNAMAN software to obtain the *β-1, 3-glucanase* full length cDNA sequence. The open reading frame (ORF) was analyzed using the NCBI ORF finder (http://www.ncbi.nlm.nih.gov/gorf/gor.html).

Plant material, bacterial strain, and vector

Tissue culture of *M. hupehensis* was amplemented by subculturing of seedlings in Murashige and Skoog (MS) medium supplemented with 2.20 mM 6-benzylaminopurine (6-BA, sigma, USA) and 0.54 mM naphthalene acetic acid (NAA, sigma, USA) and incubated under a 16 h light / 8 h dark light cycle at 25°C. Seedlings were rooted in ½ MS medium with 1.48 mM 3-indolebutyric acid (IBA, sigma, USA) 4 week. *Escherichia coli* strain DH5α cells were used to clone the full length *MhGLu* sequence and the partial sequence of *MhTubulin* was used as a housekeeping gene in real-time quantitative RT-PCR (qRT-PCR).

Plant treatments

To isolate the cDNA sequence of the *β-1, 3-glucanase* gene, seedlings rooted 4 weeks were exposed to SA (0.1 mM, Sigma, USA) for 1, 2, 4, 8, 12, 24, and 48 h, respectively, and then, 0.5 mg leaves of each treatment were mixed, frozen in liquid nitrogen, and was stored at -70°C. Seedlings rooted after 4 week were sprayed with 0.1 mM SA (Sigma, USA), 0.02 mM methyl jasmonate (MeJA, Sigma, USA), or 0.01 mM 1-aminocyclopropane-1-carboxylic acid (ACC, Sigma, USA), respectively, and *MhGLu* expression analyzed at 4, 12, and 48 h, with untreated seedlings as controls. Three seedlings were used for each treatment. Excised leaf, stem, and root were frozen in liquid nitrogen, and the tissue stored at -70°C. For apple ring spot pathogen (*Botryosphaeria berengeriana* de Not. f. sp. *Parabola* Nise Koganezawa et Sakuma) inoculation, *M. hupehensis* tissue culture seedlings were rooted after four weeks. Seedlings were cultured under controlled conditions of 90% relative humidity and an average temperature of 25°C. Abaxial leaf surfaces were sprayed with freshly collected sporangia propagated on potato dextrose agar (PDA) medium and resuspended in water at approximately 1.0×10^6 spores ml^{-1}. All leaf were collected at 0, 3, 6, 12, 24, 48, 72, and 96 h post inoculation respectively and immediately frozen in liquid nitrogen. For apple aphid (*Aphis citricota* van der Goot) treatment, in May 2010, plenty of *M. hupehensis* seedlings grown in the green house were naturally infected by the *A. citricota*. Three infected plants and three non-infected plants (controls) were selected in the same green house for the expression of *Mhchit1* gene assay. Leaves and stems were collected and frozen in liquid nitrogen and stored at -70°C.

Isolation of total RNA

Total RNA was isolated following the method by Cai et al. (2008). Total crude extract was treated with RNase-free DNase I (TaKaRa, Dalian, China) to remove genomic DNA, and RNA integrity was evaluated on ethidium bromide stained 1.0% (w/v) agarose gels. Intact 28 and 18S rRNA subunits were observed and the absence of smears on the gel indicated no RNA degradation. The concentration of total RNA was estimated by recoding absorbance at 260 nm of the samples using BioPhotometer (Eppendorf, Hamburg, Germany). The purity of RNA was verified by optical density absorption ratio of 260 /280 nm where values ranging from 1.80 to 2.00 were considered acceptably pure.

PCR amplification for the complete coding sequence of *MhGlu*

mRNA purification from total RNA was performed according to the

Oligotex™-Dt30<super> mRNA purification kit (TaKaRa, Japan). Reverse transcription of mRNA was achieved with SMART™ PCR cDNA synthesis kit (Clontech Laboratories, Inc., USA) with 0.05 µg mRNA, 1.2 µM SMART II™ A oligonucleotide and 1.2 µM 3' SMART™ CDS Primer II A primers. First strand cDNA samples were diluted 1:10 with sterile double distilled water and stored at -20°C before being used as a template for PCR amplification of *β-1, 3-glucanase*. The gene-specific primers were designed based on the assembled sequence of *MhGLu*. The PCR reaction mixture (25 µl) contained 1µl reverse transcribed first strand of cDNA and 0.4pM each of gene specific primers (BGF1: 5'-ATGGCGAAACCAAGTTCATCAGT-3' and BGR1: 5'-CGCTTAGTTGAAATTGATTGGGTAT-3'). The PCR conducted with initial denaturation step at 94°C for 5 min followed by 35 cycles at 94°C for 30 s, 55°C for 45 s, 72°C for 60 s, and a final extension at 72°C for 10 min. The PCR product was separated on 1.2% (w/v) agarose gels stained with ethidium bromide. The DNA fragment was cloned into pMD19-T (TaKaRa, China) and sequenced by the Shanghai Invitrogen Biotechnology Co. Ltd (China).

Genomic DNA PCR amplification of *MhGLu*

Genomic DNA was extracted from leaf and treated with RNase I as previously described (Tong et al. 2008). The PCR reaction mixture (25µl) contained 50 ng genomic DNA and 0.4pM each of gene specific primers (BGF1 and BGR1), and the PCR conditions were 94°C for 5 min, and then 35 cycles of 94°C for 30 s, 61°C for 45 s, and 72°C for 1.5 min, followed by 72°C for 10 min. The PCR product was ligated into the pMD19-T (TaKaRa, China) vector for sequencing.

Genome walking

Two µg genomic DNA was digested overnight with *Dra* I, *EcoR* V, *Pvu* II and *Stu* I (TaKaRa, China). The *MhGLu* promoter sequence was obtained by genome walking using the BD Genome Walker Universal Kit (Clontech, USA).

The specific primers (BGR2: 5'-GCAATAACTTCTGCTTGGGGTGGTAAG-3'; BGR3: 5'-GGCATAGTTTCTGACATTGTTTTGGAC-3') were designed based on the *MhGLu* genomic DNA sequence. The longest PCR product was cloned into pMD19-T (TaKaRa, China) vector for sequencing.

Sequence analysis

The nucleotide and deduced amino acid sequences were aligned to related genes in the GenBank database using the BLAST program and the alignment analyzed using ClustalX program. A phylogenetic tree was generated using MEGA 4.0 (Tamura et al., 2007; Kumar et al., 2008). The potential functional motifs in the promoter were analyzed using PLACE (http://www.dna.affrc.go.jp/PLACE/signalscan.html) (Higo et al., 1999; Prestridge et al., 1991), and PlantCARE (http://bioinformatics.Psb.ugent.be/webtools/plantcare/html115) (Lescot et al., 2002).

QRT-PCR

Total RNA (1 µg) was reverse transcribed with the ReverTra Ace qPCR RT kit (TOYOBO, China) for cDNA synthesis with 1 µl primer mix (random primer and oligonucleotide dT primer) and 1 µl enzyme mix (ReverTraAce reverse transcriptase and RNase inhibitor). First strand cDNA samples were diluted 1:10 with sterile double distilled water and stored at -20°C before being used as templates for real-time quantitative RT-PCR assay of *MhGLu*. Primers subjected to *MhGLu* (BGF4: 5'-ACAACAGTTGATAATGCGAGGAC-3'; and BGR4: '-GGCAAAGATGTAAGTTTCAATGGG-3') for qRT-PCR assay were designed based on the *MhGLu* cDNA sequence, and *Mhtubulin* (GeneBank accession number: GU317944) was used as a housekeeping gene using the primers (TF1: 5'-AGGTCCATCCATTGTCCACAG-3'; and TR1: 5'-TGCCAACCAAACTGACTTCAC-3'). *MhGLu* and *Mhtubulin* PCR products were cloned into pMD19-T vector and sequenced at the Shanghai Invitrogen Biotechnology Co. Ltd. The nucleotide sequences were compared with the *MhGLu* cDNA sequence and those found in GenBank using the BLASTn program.

QRT-PCR was carried out on Applied Biosystem 7300 Real Time PCR System (Applied Biosystem company, USA) with 20 µL of reaction solution, containing 1 µl of 10-fold-diluted cDNA, 0.15 pM of each primer (Invitrogen, China), 10 µl SYBR® *Premix Ex Taq*™ (Perfect Real Time) (TaKaRa, China) and 8.4 µl sterile double distilled water. The PCR conditions consisted of a 95°C denaturation for 4 min, followed by 40 cycles of 95°C for 20 s, 57°C for 20 s, and 72°C for 40 s. The relative levels of *MhGLu* compared to control *Mhtubulin* mRNAs were analyzed using the 7300 system software and the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

RESULTS

MhGLu cloning

A 1328 bp fragment containing a 1041 bp ORF was obtained by using *in silico* cloning. In order to validate the sequence, the *MhGLu* ORF sequence (GenBank accession No: FJ598140) was isolated from *M. hupehensis* and primers were designed according to the assembled sequence. The most likely cleavage site was between the 28 and 29 aa positions (S/F) as predicted by SignalP3.0 (http://www.cbs.dtu.dk/services/SignalP/). A fragment of 1396 bp from the *MhGLu* genomic DNA sequence included a 355 bp intron from start to stop codons Figure S1. The intron preserves invariant splice junction consensus sequences at the 5' (GT) and 3' (AG) ends, with the intron position similar to that of the herbaceous plant genes encoding *β*-1, 3-glucanase, such as tobacco (Linthorst et al., 1990), barley (Zeng et al., 2003), and strawberry (Shi et al., 2006). *MhGLu* encoded a 346 aa protein precursor, in which exon 1 encoded 34 aa and contained 28 aa of the signal peptide, and exon 2 encoded 312 aa. The precursor had a theoretical pI/MW of 9.42/37.80. After cleavage of the signal peptide, the predicted mature *MhGLu* had a molecular mass of 34.90 kDa and an isoelectric point of 8.75.

Species comparisons of the *MhGLu* nucleotide and deduced aa sequence

GeneBank BLAST analyses showed that the *MhGLu* coding region was most similar to a *β*-1, 3-glucanase gene from *Prunus persica* (AF435089), *Prunus avium* (EF177487) and *Vitis vinifera* (EU676806) with 84, 83, and 77% identity at the nucleotide sequence level, and

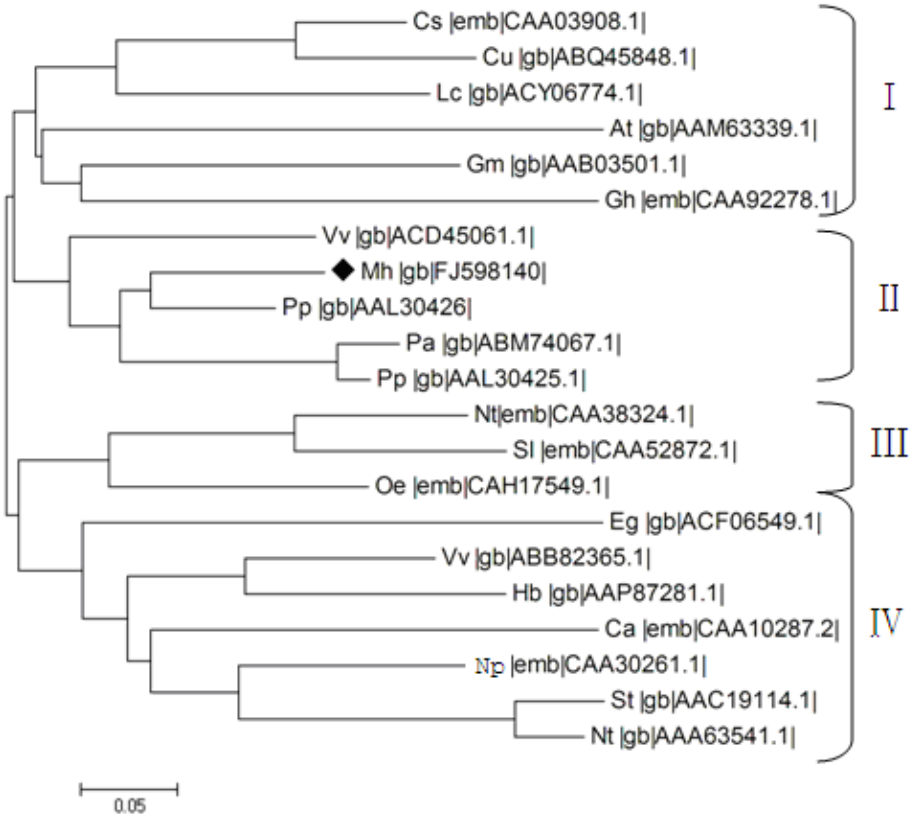

Figure 1. Phylogenetic tree of *MhGlu* and known β-1, 3-glucanase proteins in other species. GenBank accession numbers are given for each sequence following the species name abbreviations. indicates the *MhGlu* protein. Pp: *Prunus persica*; Pa: *Prunus avium*; Vv: *Vitis vinifera*; Cs: *Citrus sinensis*; Lc: *Litchi chinensis*; Gm: *Glycine max*; Oe: *Olea europaea*; Hb: *Hevea brasiliensis*; At: *Arabidopsis thaliana*; Nt: *Nicotiana tabacum*; Sl: *Solanum lycopersicum*; Gh: *Gossypium hirsutum*; St: *Solanum tuberosum*; Eg: *Elaeis guineensis*; Ca: *Cicer arietinum*; Cu: *Citrus unshiu*; Np: *Nicotiana plumbaginifolia*; Mh: *Malus hupehensis*.

84% (AAL30426), 74% (ABM74067) and 76% (ACD45061) identity at the aa sequence level, respectively. *MhGlu* was classified as a family 17 glycohydrolase Figure S2.

We also searched 20 different *β*-1, 3-glucanase genes from different plant species through BLASTP. Plant *β*-1, 3-glucanases can be grouped into four clusters (Figure 1). *MhGlu* in *P. persica*, *P. avium* and *V. vinifera* was closely related and fell within the same cluster, suggesting that woody plants *β*-1, 3-glucanases may have similar functions compared to herbaceous plants.

Cis-element analysis of the *MhGLu* promoter

In order to analyze whether *MhGLu* was regulated by SA, MeJA, and ACC, the translation start codon was designated as +1, and about 1.5 kb upstream genomic DNA sequence of the *MhGLu* gene was cloned from *M. hupehensis* genomic DNA through genome walking. PlantCARE and place analysis of the *MhGLu* promoter showed that there were a number of regulatory elements in response to environmental signals (Table 1).

Five kinds of potential hormone binding elements were identified, including the SA motif with WBOXATNPR1 (TTGAC), MeJA-responsive element with CGTCA-motif (CGTCA), ethylene responsive element with LECPLEACS2 (TAAAATAT), gibberellin-related GARE-motif (AAACAGA), and part of an auxin-responsive element with TGA-box (TGACGTAA). Furthermore, the promoter region contained several other putative regulatory motifs known to respond to abiotic stress. These motifs were MBS (TAACTG), the TC-rich repeats (ATTCTCTAAC), CBFHV (RYCGAC), LTRE1HVBLT49 (CCGAAA), and WBOXNTERF3 (TGACY). MBS (TAACTG) is a binding sequence involved in dehydration stress-response and the TC-rich repeats (ATTCTCTAAC) involved in defense and stress response. The CBFHV (RYCGAC) is involved in dehydration-responsive element (DRE) binding proteins, and LTRE1HVBLT49 (CCGAAA)

Table 1. Cis-elements of *MhGlu* promoter.

Cis-element	Sequence(5'-3')	Function	Database
TGACG motifs	TGACG	Salicylic acid- and auxin-responsive element	Place
Box-W1	TTGACC	Fungal elicitor responsive element	Plantcare
CGTCA-motif	CGTCA	Cis-acting regulatory element involved in the MeJA-responsive	Plantcare
GARE-motif	AAACAGA	Gibberellin-responsive element	Plantcare
MBS	TAACTG	MYB binding site involved in drought-inducibility	Plantcare
TC-rich repeats	ATTCTCTAAC	Cis-acting element involved in defense and stress responsiveness	Plantcare
TGA-box	TGACGTAA	Part of an auxin-responsive element	Plantcare
CBFHV	RYCGAC	Dehydration-responsive element (DRE) binding proteins	Place
LECPLEACS2	TAAAATAT	Ethylene -responsiveness	Place
LTRE1HVBLT49	CCGAAA	Low-temperature-responsive element	Place
WBOXATNPR1	TTGAC	Salicylic acid (SA)- responsive element	Place
WBOXNTERF3	TGACY	Wounding- responsive element	Place

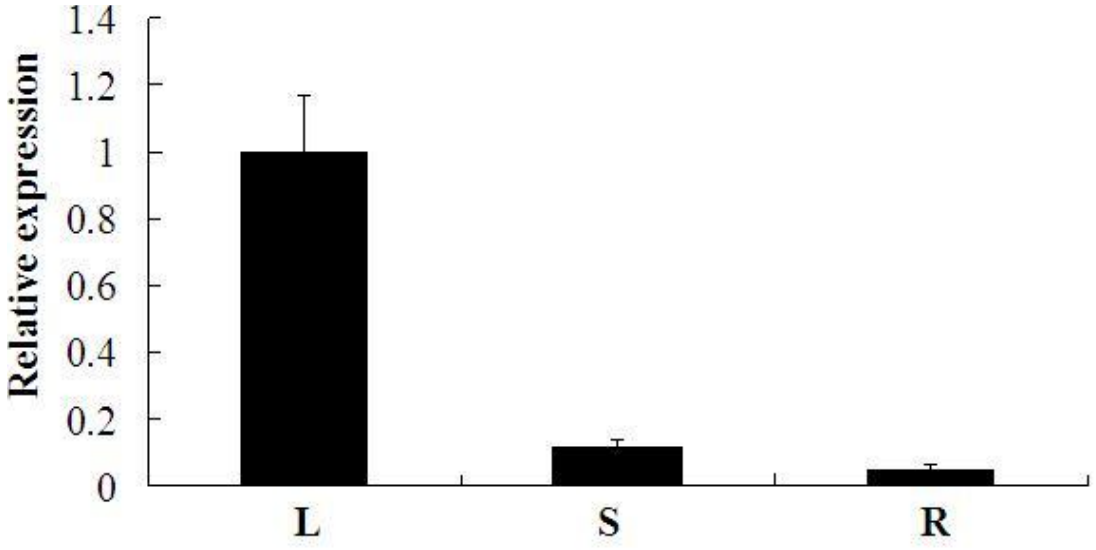

Figure 2. *MhGlu* expression analysis in different leaf (L), stem (S), and root (R) in *M. hupehensis*. Data were normalized to the *Mhtubulin* expression level. The mean expression value was calculated for three independent replicates. Vertical bars represent the standard deviation.

is the low-temperature-responsive element. WBOXNTERF3 (TGACY) is involved in the wound-related activation of the tobacco ERF3. The promoter region also contained the biotic-responsive element, Box-W1 (TTGACC), which is a fungal elicitor responsive element. The presence of these potential regulatory motifs suggests that *MhGLu* may be involved in regulation of plant tolerance to several types of stresses.

Expression pattern of *MhGlu* in various tissues of *M. hupehensis*

To study the expression pattern of *MhGlu* gene in various tissues (leaf, stem and root) of *M. hupehensis*, qPCR were performed using gene specific primers. The *MhGlu* leaf basal expression level was about 9 times higher than in stem, and 20 times higher than in root (Figure 2). Previous studies showed that *β-1, 3-glucanase* was present in leaf, peel, and pulp of plantains, but not in the root and corm (Jin et al., 2007). A study by Shi et al. (2006) showed that the *FaBG2-1* and *FaBG2-3* were constitutively expressed in the leaf, crown, root, and fruit, and the expression of the two genes in leaf was higher than in the root. Like in strawberry and plantains, the obvious expression differences in tested tissues may be related to plant tissue specific pattern of *MhGLu* expression.

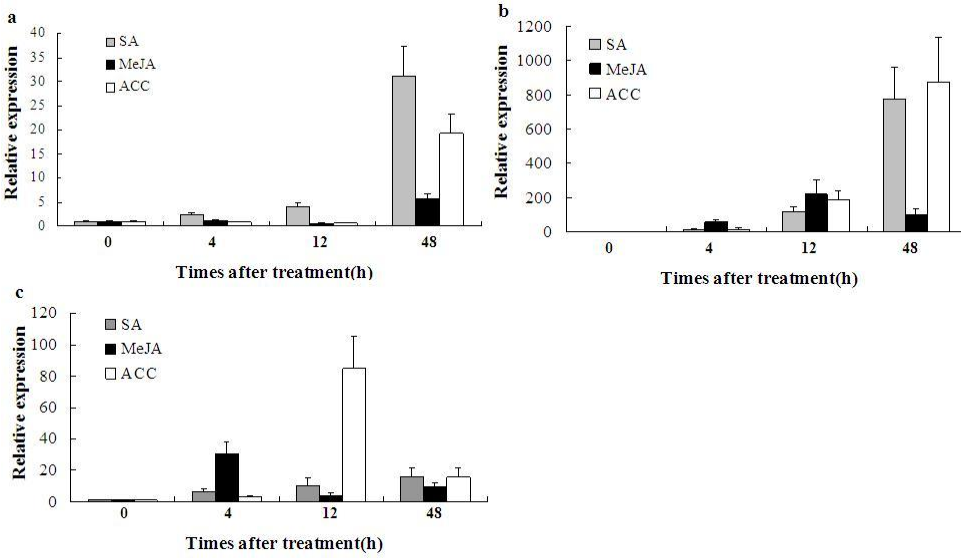

Figure 3. *MhGlu* expression analyses in *M. hupehensis* tissue culture seedlings treated with SA, MeJA, and ACC using real-time quantitative RT-PCR *in* leaf (a), stem (b), and root (c).Data were normalized to the *Mhtubulin* expression level. The mean expression value was calculated for three replicates. Vertical bars represent the standard deviation.

MhGLu expression in response to SA, MeJA, and ACC

Our result showed that the SA responsive motif, MeJA and ethylene responsive elements were included in the promoter sequence of *MhGLu*. QRT-PCR were performed using gene specific primers BGF4 and BGR4 and *Mhtubulin* as a housekeeping gene to further check whether the *MhGLu* transcript accumulated in *M. hupehensis* after treatment with SA, MeJA, and ACC. *MhGlu* expression was induced by SA, MeJA, and ACC in all the treated tissues including leaf, stem, and root (Figure 3).

When plants were treated with SA, *MhGLu* expression was increased at 4 h (2.41-fold) and reached the peak at 48 h (31.11-fold) in leaf of *M. hupehensis*. No change of *MhGLu* accumulation was visible during the first 12 h and was increased at 48 h (5.73 -fold for MeJA and 19.16-fold for ACC) in leaf of *M. hupehensis* after treatment with MeJA and ACC (Figure 3a). *MhGLu* expression in stem was higher at 4 h (9.84-fold for SA and 11.10 – fold for ACC), 12 h (113.06 -fold for SA and 182.66 – fold for ACC) and reached the peak at 48 h (772.29 - fold for SA and 871.89 – fold for ACC) after SA and ACC treatment. *MhGLu* expression in this tissue was increased significantly at 4 h (15.47 -fold), and reached the peak at 12h (85.17- fold) followed by a decline when treated with MeJA (Figure 3b). *MhGLu* transcript accumulation in root was increased at 4 h (6.02 - fold), 12 h (9.90 - fold) and reached the peak at 48 h (16.24 - fold). *MhGLu* expression reached a peak at 4 h (30.42-fold) and declined after in root treated with MeJA. In the ACC treatment, the *MhGLu* expression was enhanced at 4 h (2.79 - fold) and reached peak at 12 h (84.84- fold, Figure 3c). These results showed that the *MhGlu* gene was differentially regulated by the inducers applied in various tissues of *M. hupehensis*.

MhGLu expression in response to biotic stresses

QRT-PCR was performed to confirm whether *MhGLu* was induced in *M. hupehensis* leaves after fungal attack (Figure 4a). *MhGLu* transcription was not induced during the first 12 h post inoculation (hpi) with apple ring spot pathogen, but was up-regulated at 24 hpi(2.52 - fold) and peaked at 48 hpi (8.52 - fold). *M. hupehensis* seedlings grown 2 year old were infected with apple aphids in green house. Three seedlings were randomly selected for gene expression analysis and three uninfected seedlings were used as controls. *MhGLu* gene expression levels were enhanced in young leaf and stem infected with the apple aphid (Figure 4b).

DISCUSSION

This is the first report on the isolation of *β-1, 3-glucanase* gene sequence from *M. hupehensis* and study of its expression pattern in tissues including leaf, stem and root after exposure to hormones (SA, MeJA and ET), the apple ring spot pathogen and the apple aphid. Like other plants (Jin et al., 2007; Liu et al., 2010), the deduced aa sequence of *MhGLu* contained two district, one sequence

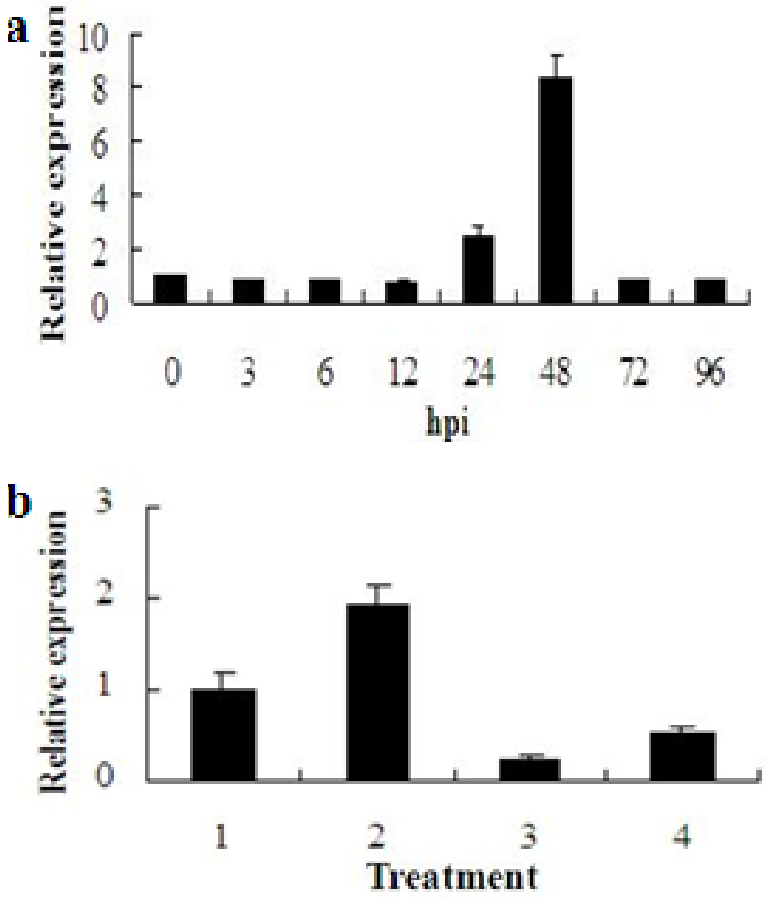

Figure 4. QRT-PCR analyses of *MhGlu* expression profiles in *M. hupehensis* infected with apple ring spot pathogen (a) and apple aphid (b). Hpi: hours post infection. QRT-PCR analyze of *MhGlu* expression profiles in *M.hupehensis* in leaf (1, 2) and stem (3, 4) with (2, 4) or without (1, 3) apple aphid infection. Data were normalized to the *Mhtubulin* expression levels. The mean expression value was calculated for three replicates. Vertical bars represent the standard deviation.

was an N-terminal signal peptide (No. 1-28 aa), and the other with glycosidase hydrolase family 17, [LIVMKS] - x - [LIVMFYWA] 3 - [STAG] - E – [STACVI] - G - [WY] - P - [STN] - x - [SAGQ], was LeIVISESGWPTaG (No.264-277 aa), where *E* is an active site residue *Glu*. The MhGLu protein has the same structural feature as the *β*-1, 3-glucanase gene from herbaceous plants, like wheat，plantain, suggesting that the *β*- 1, 3-glucanase gene may be conserved in function.

Two types of resistance have already been recognized based on signaling pathways including induced systemic resistance (ISR) and SAR (Durrant and Dong, 2004). In plants, SA and JA/ET are important regulators of these two induced defense responses, respectively (Koornneef and Pieterse, 2008; Spoel and Dong, 2008; Leon-Reyes et al., 2009). *β- 1, 3-glucanase* is generally known as one of the pathogenesis -related protein gene downstream components of the SAR pathway (Liu et al., 2010). In wheat, *TaGlu* was induced by SA, MeJA, and ET, suggesting that *β- 1, 3-glucanase* might be involved in both SAR and ISR (Liu et al., 2010). In this study, SA motif, MeJA- and ethylene responsive elements were found in the promoter of *MhGLu* gene, and expression analysis of *MhGLu* in various *M. hupehensis* tissues showed that SA, MeJA, and ET treatments all enhanced *MhGlu* expression in leaf, stem, and root. These results suggested that *MhGlu* might be involved in both SAR and ISR in *M. hupehensis*.

Comparison of the level of expression of the *MhGLu* in various tissues treated with SA, MeJA and ACC revealed that the expression was regulated differently even though these hormones could induce the gene expression. Enhanced expression was obvious at 4 h and reaches the peak at 48 h in all studied tissues after SA treatment. However, the ratio of increasing expression in stem was higher than in leaf and root. The expression patterns of

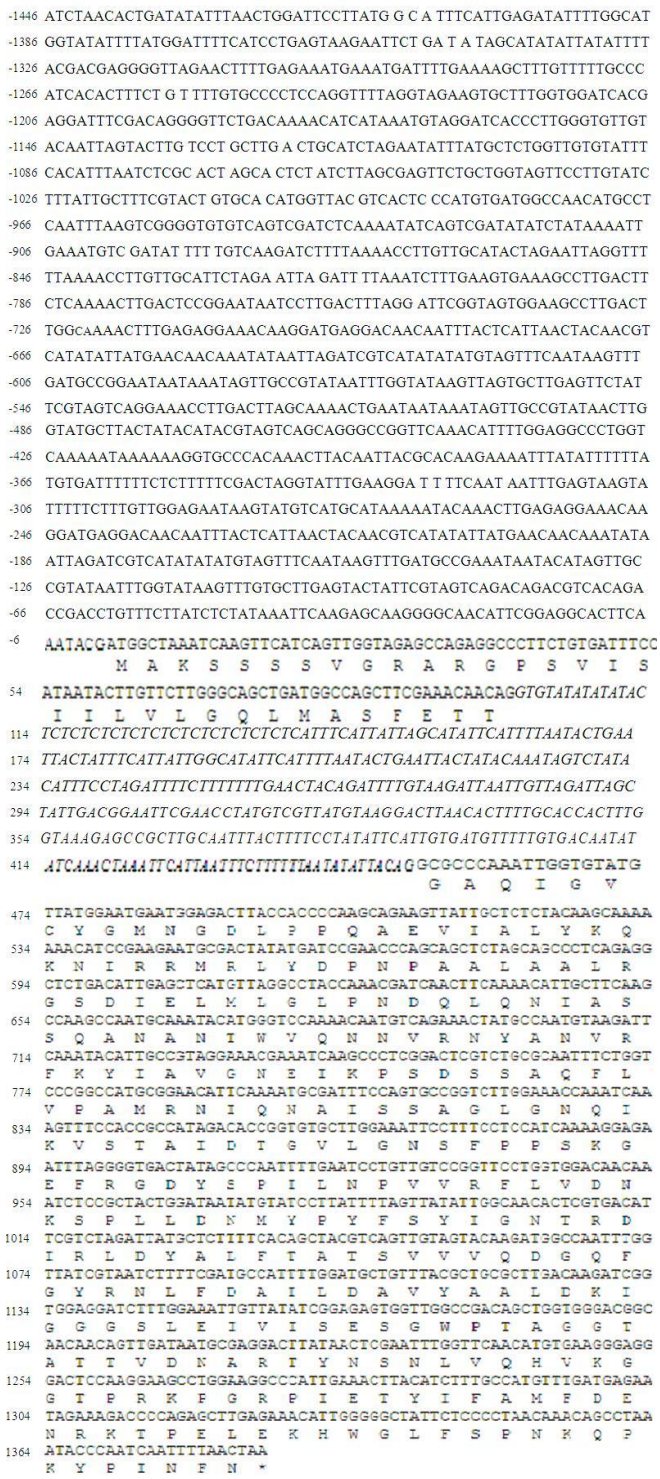

Figure S1. *MhGlu* nucleotide sequence of genomic DNA and its promoter sequence. The coding region is separated by an intron (italics). * indicates the stop codon.

MhGLu gene in all studied tissues treated by MeJA were different. The results suggested that the *MhGLu* gene in root was more responsive to MeJA than in leaf and stem. But the ratio of expression increased in stem was also higher than in leaf and root. The expression profile for ET treatment in leaf was similar to MeJA treatment and in

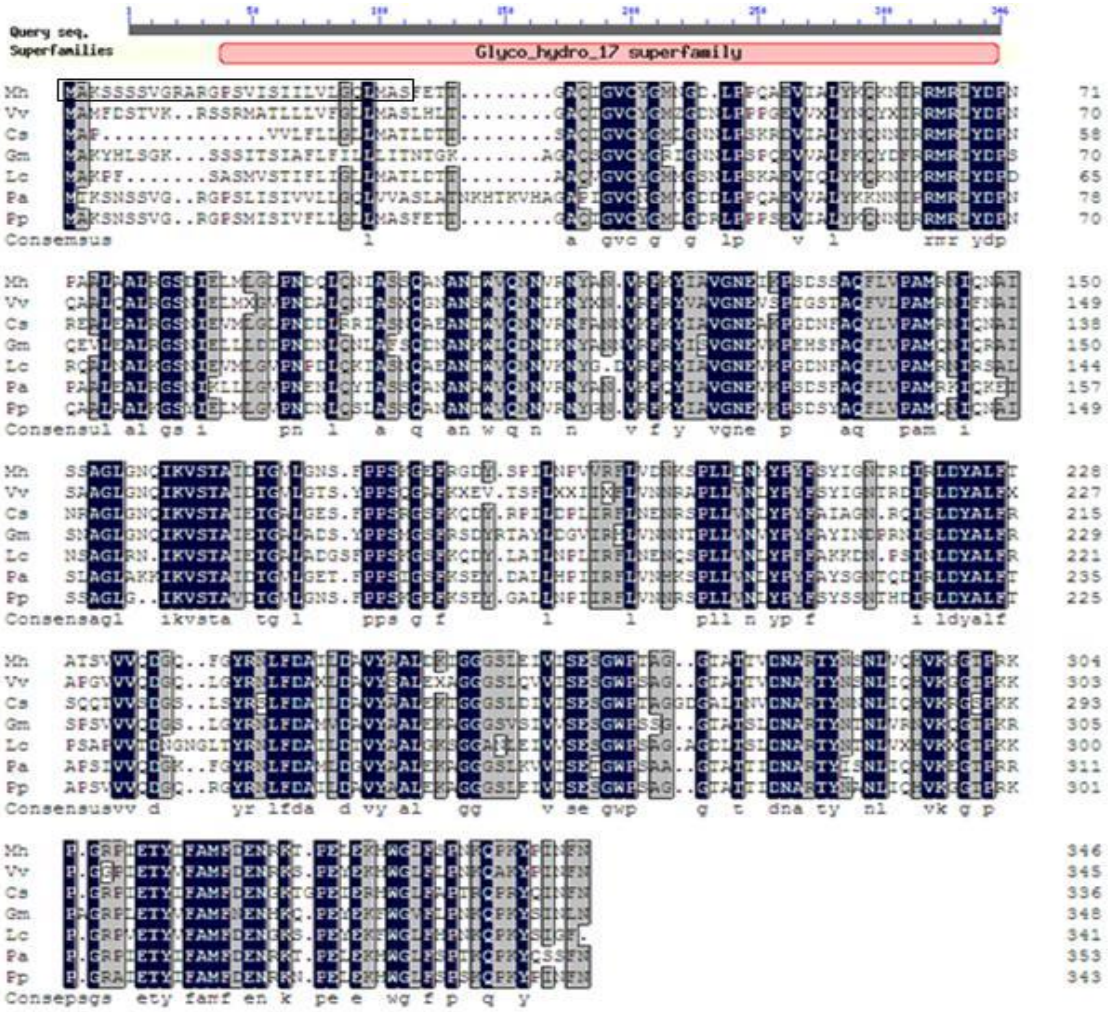

Figure S2. Comparison of the *MhGlu* deduced aa sequence with the sequences of related proteins: Pp = *Prunus persica* (GeneBank accession: AL30426); Pa = *Prunus avium* (GeneBank accession: ABM74067); Vv = *Vitis vinifera* (GeneBank accession: ACD45061); Cs = *Citrus sinensis* (GeneBank accession: CAA03908); Lc = *Litchi chinensis* (GeneBank accession: ACY06774); Gm = *Glycine max* (GeneBank accession: AAB03501); Mh = *Malus hupehensis*. *MhGlu* fully conserved residues (black shade), semi-conservative substitutions (gray shade), and the signal peptide (paned) are indicated.

stem was similarity to SA treatment. However, it had special expression in root. Enhanced expression was found and reaches the peak at 12 h. Our dates indicated that the *MhGLu* gene was expressed in leaves, stems and roots, and can be differentially regulated by inducers studied, suggesting that *MhGLu* can play its role in different tissues. Given the diversity of expression patterns for *MhGLu* detected in different organs, *MhGLu* appears to have tissue specific expression pattern.

The results from gene expression studies showed that no or low levels of induction of the *MhGLu* gene occurred at the early time point after infection with apple ring spot pathogens. However, higher level transcript accumulation was found at the 24 and 48 h time points. These results are in agreement with previous conclusion that *FaBG2-1* and *FaBG2-2* was higher induced by infection with *C. fragaiae* or *C. acutatum* at the 24 or 48 h in leaf of strawberry, and *Glu1* was high induced by inoculation with *C. laurentii* at 24 and 48 h (Shi et al., 2006). It was possible that the accumulation of *MhGlu* gene from 24 to 48 hpi may related to the invasion process exhibited by the fungi or deprivation of available nutrients. *MhGLu* gene was induced by the apple aphid in leaf and stem compared to the non-infected plants. It was possible that the apple aphid induced the expression of *MhGLu* gene. These results showed that *β*-1, 3-glucanase, which is a pathogenesis-related protein gene, might be playing a key role in defense responsive against fungi and insects in *M. hupehensis*.

To our knowledge, this is the first implementation of cloning the *β*-1, 3-glucanase from *M. hupehensis*. *MhGlu*, which was one of important *PR* genes, might be involved

in both SAR and ISR, and might be concerned with defense responsive against fungi and insects in *M. hupehensis*. In future studies, we will concentrate on transformation the *MhGlu* gene into the 'Fuji' apple to determine if this could enhance resistance to apple pathogens (apple ring spot, apple powdery mildew and apple canker pathogens) and insects (apple aphids).

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Abbreviations: SA, Salicylic acid; **MeJA,** methyl jasmonate; **ET,** ethylene; **ACC,** 1-aminocyclopropane-1-carboxylic acid; ***NPR*1,** non-expresser of pathogenesis related genes1; **MS,** Murashige and Skoog; **PI,** post inoculation; **QRT-PCR,** real-time quantitative RT-PCR; **PI,** post inoculation; **SAR,** systemic acquired resistance; **PR,** pathogenesis related; **EST,** expression sequence tag; 6-BA, 6-benzylaminopurine; **IBA,** 3-indolebutyric acid; **PDA,** potato dextrose agar; **ISR,** induced systemic resistance; **Hpi,** hours post infection.

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