

Molecular Cloning, Characterization and Expression Analysis of *MhRAR1* Gene from *Malus Hupehensis*

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Abstract: A novel *RAR1* gene, designated *MhRAR1*, was cloned by the methods of RT-PCR and RACE from *Malus hupehensis*. The full length sequence of *MhRAR1* is 1065 bp with an open reading frame of 678 bp, encoding a protein of 225 amino acids. As found in other plant *RAR1* proteins, sequence alignment showed that *MhRAR1* protein contains two CHORD domains and one plant-specific CCCH domain. In addition, the *MhRAR1* contains conserved strings of invariant cysteine and histidine residues within the CHORD domains and CCCH domain. These results suggested that *MhRAR1* protein from *M. hupehensis* might share the similar function with the *Arabidopsis thaliana* *RAR1* and *Hordeum vulgare* *RAR1*, and is an important component of R gene-mediated disease resistance. Phylogenetic analysis revealed that *MhRAR1* was closely related to *Ricinus communis* *RAR1*. The analysis by qRT-PCR revealed that the expression of *MhRAR1* gene was higher in leaves than that in stems and roots. SA, MeJA and ACC treatment induced *MhRAR1* expression in stems and roots, but not in leaves. Expression of *MhRAR1* was weakly induced in *M. hupehensis* after infection with *Botryosphaeria berengeriana*. The cloning and characterization of the *MhRAR1* gene will be useful for further studies of biological roles of *MhRAR1* in plants.

Keywords: *Malus hupehensis*, *MhRAR1*, cDNA Cloning, Expression Pattern

1. Introduction

Plants are frequently attacked by various disease pathogenesis with complicated strategies colonizing their hosts, and have evolved defense mechanisms to protect themselves effectively from microbial pathogens. Plants various resistance proteins (R proteins) activate resistance responses including rapid ion fluxes, generation of reactive oxygen species (ROS), production of antimicrobial compounds, and the accumulation of salicylic acid (SA) which was crucial for the induction of systemic acquired resistance (SAR). These responses are accompanied by the hypersensitive response (HR), localized programmed cell death at the site of pathogen invasion [1].

RAR1 (Required for *Mla12* resistance), which was a crucial component of resistance conferred by many R proteins [2,3], play a key role in disease resistance of plants. *Arabidopsis rar1* mutants are defective in R-protein mediated resistance against several bacterial (*Pseudomonas*) and oomycete (*Peronospora*) pathogens [4,5]. *Rar1* mutants fail to accumulate ROS or mount the HR in barley [6]. In the past several decades, many studies have been done to discover the molecular function of

RAR1 protein in plants. Numbers of researches have been carried out in the model plants such as *Arabidopsis* [4,7,8], barley[3, 9-11], tobacco[12] and so on. However, none of work was done in woody plants especially in fruit trees such as apple.

The plant hormones SA, MeJA and ET are three types of signalling mediators that activate the plant defence response against pathogen attack [13,14]. In general, SA and JA/ET are thought to regulate two different signal transduction pathways in plant disease resistance. SA and JA/ET signalling pathways and the cross-talk between them have been studied in various plants [15-17]. To determine whether exogenous application of these stimuli could induce the expression of *MhRAR1* gene, *Malus hupehensis* were treated with SA, MeJA and ACC.

Malus hupehensis, common names *Hupeh* crab or tea crabapple, is a species of flowering plant in the apple genus *Malus* of the family *Rosaceae*, native to Japan and Taiwan and province of China. It is a vigorous deciduous tree growing to 12 m (39 ft) tall and broad, with pink buds opening to fragrant white blossom in Spring, and bright red, cherry-sized

crab-apples in the Autumn. *M. hupehensis* was an apple rootstock that is tolerant to various apple pathogens [18]. Here, we isolated and functionally characterised of a *M. hupehensis* *RAR1* gene. To better understand *MhRAR1* gene functions, we also analysed the expression of *MhRAR1* in response to plant hormones, such as SA, MeJA, and ACC in different tissues of *M. hupehensis*, and in response to biotic stress apple ring spot pathogenesis *Botryosphaeria berengeriana*.

2. Materials and Methods

2.1. Plant Materials and Bacterial Strains

M. hupehensis plants were subcultured in vitro in Murashige and Skoog (MS) medium supplemented with 6-benzylaminopurine (6-BA, 0.5mg/L) and naphthalene acetic acid (NAA, 0.1mg/L) and cultured under a 16-hour light (25°C)/8-hour dark (25°C) cycle. *Escherichia coli* strain DH5 α cells were used for the cloning of *MhRAR1*, and propagation of all the recombinant plasmid vectors. The Pyk2478 vectors were used for the construction of plant expression vectors of the *RAR1* gene.

2.2. Treatment of *M. hupehensis* with SA, MeJA and ACC and Apple Ring Spot Pathogen

Three weeks old *M. hupehensis* in vitro plant leaves were sprayed with 0.1mM SA (Sigma), 0.02mM MeJA (Sigma) and 0.01mM ACC (Sigma) for 4, 12 and 48h, respectively, with water treatment as a control. Each treatment group consisted of three plants. Then, the leaves, stems and roots were harvested and frozen in liquid nitrogen. Tissues were stored at -70°C until use. For apple ring spot pathogen inoculation (*B. berengeriana*), *M. hupehensis* tissue culture plants were rooted after four weeks. Abaxial leaf surfaces were sprayed with freshly collected sporangia propagated in potato dextrose agar (PDA) media and resuspended in water at approximately 1.0×10^6 spores·mL⁻¹. Leaves were collected at different time points and immediately frozen in liquid nitrogen.

2.3. Extraction of Total RNA and Clone of *MhRAR1*

PCR was performed with the primers RF1 and RR1 (Table 1) derived from the conserved sequence region of *RAR1* using a complete cDNA library [19] in order to isolate a partial cDNA fragment (554bp) of the *MhRAR1* gene. Then, using a sequence obtained from the initial cDNA fragment, new gene-specific sense and antisense primers were designed for the 5'- and 3'- rapid amplification of cDNA ends (RACE) reactions (Table 1). The missing 3' and 5' sequences were obtained by RACE with SMARTTM RACE cDNA Amplification Kit (Clontech Laboratories, Inc. No. 634902). The conserved, 3' and 5' sequences were assembled using the DNAMAN software. To verify the assembled sequence, PCR was performed using the primers RF3 and RR3 (Table 1) from the complete cDNA library to amplify the open reading frame (ORF) of *MhRAR1*. Genomic DNA was extracted from leaves and treated with RNase I as described by Tong et

al.[20]. *MhRAR1* gene cloning from genomic DNA was performed as above using the primers RF3 and RR3 (Table 1). The PCR products were cloned into a pMD19-T vector (TaKaRa) and sequenced by the Shanghai Invitrogen Biotechnology Company Ltd.

The amino acids sequence of *MhRAR1* was deduced and analyzed using ProtParam tool (<http://cn.expasy.org/tools/protparam.html>). Nucleotide and protein sequence similarity analyses were carried out at the NCBI server using BLAST programs (<http://www.ncbi.nlm.nih.gov/BLAST/>). Amino acid alignment sequences of *RAR1* homologues were downloaded from GenBank and the alignment of the amino acid sequences with that of *MhRAR1* was performed using BioEdit software. A comparative phylogenetic tree was then constructed using MEGA software version 4.0 with the Neighbour-Joining method [21] and 1000 replicates were used for bootstrap test. The tree was then visualised using the tree view package[22].

2.4. Gene Expression Analysis of *MhRAR1* in *M. hupehensis* Using Real-Time Quantitative RT-PCR (qRT-PCR)

Total RNA isolation were performed as described by Cai et al. [23] and DNase I treatment was performed according to the manufacturer's protocol. First strand cDNA was synthesised with the ReverTraAce qPCR RT Kit (TOYOBO, Code No.: FSQ-101) according the manufacturer's instructions. QRT-PCR analyses with a pair of primers (Table 1) were performed to determine the tissue-specific expression (leaf, stem and root) and expression profiles of *MhRAR1* after various treatments. Normal PCR reactions were performed to ensure gene-specific amplification using the primers BRF1 and BRR1 to amplify the *MhRAR1* gene. A single PCR fragment with the expected size was amplified, suggesting that the primers were suitable for qRT-PCR analyses. To confirm the expected fragment of the *MhRAR1* gene, the resulting PCR product was cloned and sequenced. All samples were harvested and three biological replicates were run independently. *M. hupehensis* tubulin was amplified with a pair of primers and used as the housekeeping gene for the qRT-PCR analyses. Templates were the 10 \times diluted cDNAs from each sample. The reaction protocol was as follows: 1 μ L of 10-fold diluted cDNA, 0.3 μ L 10pM of each primer (Table 1), 10 μ L SYBR® Premix Ex Taq™ (Perfect Real Time) (TaKaRa Code: DRR041A) and 8.4 μ L of sterile double distilled water. The thermal conditions were: 95°C for 4 min, 95°C for 20 s, 58°C for 20 s and 72°C for 43 s for 40 cycles. Dissociation curves from 55°C to 95°C were generated for each reaction to ensure specific amplification, along with verification by gel electrophoresis. The qRT-PCRs were performed using a 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The relative levels of genes respective to control Mhtubulin mRNAs were analysed using the 7300 system software and the 2^{- $\Delta\Delta$ Ct} method [24]. Data analyses were conducted using SPSS version 17.0 statistical software. For all analyses, the level of significance between different time points was set at P<0.05.

2.5. Subcellular Localisation of the MhRAR1-EGFP Fusion Protein

Subcellular localisation of MhRAR1 was assayed according to the method of Wang et al.[25]. For generating the MhRAR1-GFP fusion construct, the *MhRAR1* ORF was amplified with a forward primer YXRAF1 containing a

BamH I site and a reverse primer YXRAR1 containing an *EcoR I* site (Table 1). The resulting PCR products were cloned into the pMD19-T simple vector (TaKaRa) and sequenced. The entire ORF of the *MhRAR1* gene was inserted into the plasmid Pyk2784-EGFP which included enhanced green fluorescent protein.

Table 1. Primers used for this paper.

Primers name	Sequence [5'-3']	Usage
RF1	ACTTGCGACGCCATGTTC	Cloning of MhRAR1 conserved sequence
RR1	TCGCAACATTTCCACCCTC	
RF2	AGAGAAAGATAACCACGAGAATGC	3'RACE
RR2	GGAGCAGAAGTGCCTTGATCGTCAGTAAT	5'RACE
RF3	ACACTAGTGGATGGAGGGTC	PCR amplification of MhRAR1 ORF
RR3	GGTACCTTAAGATACCGGGT	
BRF1	TGATATAAACCCAGCCCCAGACG	
BRR1	CACAGCACTTCCACCCTC	Expression analysis for MhRAR1
BAF1	AGGTCCATCCATTGTCCACAG	Housekeeping gene
BAR1	TGCCAACCAAACTGACTTCAC	
YXRAF1	ACGGATCCGGATGGAGGGTC	
YXRAR1	AGAATTCAGATACCGGGT	Vector construction of Subcellular localization

3. Results

3.1. Analysis of the MhRAR1 Gene Sequence and the Predicted Protein

(a)

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1 GACCTGTGGACTTCCAGTCAGTCTTTGTTTCTGGTCTTCTCCCGCGGTCGTCCTC
61 CCTCCGGCGTCTCTTCTCTGTTCCCTTCTCTCTTTTCTGGTATTTTCTG
121 GGCAGACACAGAGCCGACAGATGGATGGAGGGTACCGGGCAGCTCTGTTTCGATCG
      M E G Q R A A L F R C
181 CAGCGAATTGGCTGCAACGCCATGTTTCCGAGGACACAATCCGAAGGCTCCTGTCAA
      Q R I G C N A M F S E D D N P E G S C Q
241 TACCACGATTCGGGACCTATATTTTCATRATGGGATTAAGAGTGGAGTTGTTGCAAGAAA
      Y H D S G P I F H X G I K E W S C C K K
301 AGAAGTCATGATTTACCTTATTTTAGAATTACCAGGATGTAAGACAGGTAACACACA
      R S H D F T L F L E L P G C K T G K H T
361 ACAGAGAAACAGTGTGGCGAAGCCGAAGCTCAAGGCTCCTGTCTCTCTACTGCA
      T E K P V L A K P K P K A P V P A P T A
421 GCTCTGCTACTGATGCATCGTCAAAACAGTCTGCTCTAGGTGTCGCCAGGGTTTTTTC
      A P A T D A S S K Q S C S R C R Q G F F
481 TGTTCAGAACATGGTGCACAAGCCAAAGAACTAAAATCAAGCCGTGAAAATGGGAAACA
      C S E H G A Q A K E L K S K P V N V E T
541 ACTCCAAITCCAGAAAGCATTACTGACGATCAAGGCACITCTGCTCCGCCGAAAAGATT
      T P I P E S I T D D Q G T S A P P K K I
601 GTTGATATAAACCCAGCCGACGCTGTATGAATAAGGGGTGTGATCAAACITTTCAAAGAG
      V D I N Q P Q T C M N K G C D Q T F K E
661 AAAGATAACCCAGAGATGCAATGCAATGCAATGCAATGCAATGCAATGCAATGCAATGCA
      K D N H E N A C S Y H P G P A I F H D R
721 GTGAGAGGGTGGAAAGTGTGTGACGTTACGTAAGGAATTTGATGAGTTTATGAGCATT
      V R G W K C C D V H V K E F D E F M S I
781 CCTCATGCACCAAGGTTGGCACAATGCTGACCCGGTATCTTAACATCAGATGCAGGGA
      P P C T K G W H N A D P V S *
841 AGGATGGTGTTTTACCCTGCTTTTGTGTGAAATTTCTTTTGTATTGAAGTAAAA
901 TACATTTCTGCAACTGCTCTCCAGGAATTTAGCTTCTCTGATACACCTTAGAAT
961 ATATGAATTGCTTGTCTGTGTTCTTGTGAAATTTGAACAAAATTCGTTGTGGATATC
1021 AAACCTTTTACGGCTCTAAAAAATAAAAAAAAAAAAAAAAAAAAAA
  
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(b)

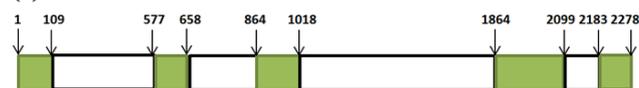


Fig. 1. The structure of *MhRAR1* gene and protein. (a) The nucleotide and deduced amino acid sequences of cloned *MhRAR1*. The protein sequence is indicated by one letter code below the nucleotide sequence. * indicate the stop codon. (b) Schematic diagram of *MhRAR1* gene. Reseda indicated the exons and White indicated the introns.

A full-length 1,065 bp of *M. hupehensis RAR1* was cloned using the methods of RT-PCR and RACE. It contained an

open reading frame (ORF) of 678 bp encoded a protein of 225 amino acids. The cDNA had a 5'-untranslated region (UTR) of 147 nucleotides, a 3'-UTR of 240 nucleotides including a stop codon (TAA) (Fig. 1a). The calculated molecular mass was 24.90 kDa, and the estimated isoelectric point (pI) of this protein was 7.51. The *MhRAR1* cDNA sequence has been submitted to the NCBI GenBank as accession number FJ593502. The *MhRAR1* genomic DNA sequence from start to stop codon was 2278 bp, and there were five exons and four introns in the complete coding region of *MhRAR1* when the cDNA sequence and genomic DNA sequence were compared (Fig. 1b).

3.2. Multiple Sequences Alignment and Phylogenetic Analysis

A search in the GenBank database, using the BLASTP program, revealed that the predicted MhRAR1 deduced amino acid of *M. hupehensis* exhibited high similarities with those of the *M. domestica* (identities 97 %), *Fragaria vesca subsp. vesca* (identities 84 %), *Prunus mume* (identities 84 %) and *Ricinus communis* (identities 74 %). Similar to *A. thaliana* [4], barley and tobacco [12], there were two cysteine and histidine-rich Zn²⁺-binding domains (designated as CHORD) and one plant-specific conserved Cys residues and a His residue (CCCH) domain in MhRAR1 protein (Fig.2). In addition, the MhRAR1 contains conserved strings of invariant Cys and His residues within the CHORD domains (Fig.2). The amino acid sequences outside the CHORD and CCCH domains are significantly different among plant PAR1 proteins (Fig.2). To determine the phylogenetic relationship between MhRAR1 and other plant RAR proteins, a phylogenetic tree was constructed, revealing that MhRAR1 was closely related to MdRAR1, FvRAR1 and PmRAR1 (Fig. 3).

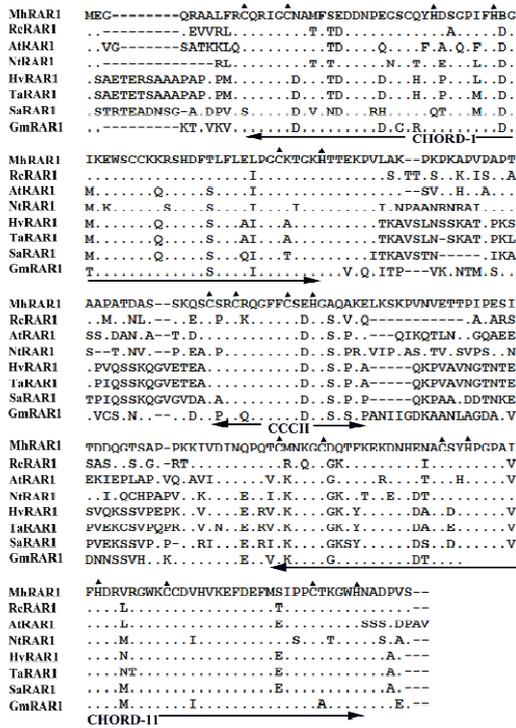


Fig. 2. Amino acid sequence comparison of the predicted *Malus hupehensis* RAR1 protein with the other plant species RAR1. CLUSTALW produced alignment was formatted using BioEdit software. Conservative substitutions are shaded in dot mark. Two conserved CHORD domains (CHORD-I and II) and one conserved CCCH domain are shown. Triangle (▲) indicate invariant conserved cysteine and histidine residues within the CHORD and CCCH domains. The accession numbers of plant RAR sequences are as follows: *MhRAR1*: *Malus hupehensis* RAR1 (gbACT98264); *RcRAR1*: *Ricinus communis* RAR1 (ref XP002510896); *AtRAR1*: *Arabidopsis thaliana* RAR1 (dbjBAB11239); *NtRAR1*: *Nicotiana tabacum* RAR1 (gbAAM62409); *HvRAR1*: *Hordeum vulgare* RAR1 (gbAAF18432); *TaRAR1*: *Triticum aestivum* RAR1 (gbABN13684); *SaRAR1*: *Saccharum hybrid cultivar* RAR1 (gbACX68653); *GmRAR1*: *Glycine max* RAR1 (gbACI31548).

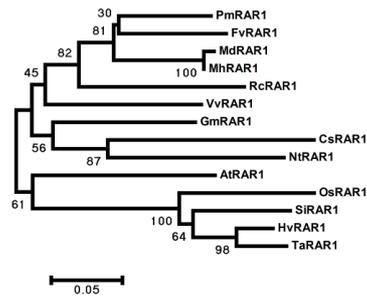


Fig. 3. Phylogenetic relationships of *MhRAR1* with other plant RAR1. Phylogenetic tree was constructed using MEGA software version 4.0 with the Neighbour-Joining method (Tamura et al. 2007) and 1000 replicates were used for bootstrap test. The tree was then visualised using the tree view package (Page 1996). The accession numbers of plant RAR sequences are as follows: *MhRAR1*: *Malus hupehensis* RAR1 (gbACT98264); *RcRAR1*: *Ricinus communis* RAR1 (ref XP002510896); *AtRAR1*: *Arabidopsis thaliana* RAR1 (dbjBAB11239); *NtRAR1*: *Nicotiana tabacum* RAR1 (gbAAM62409); *HvRAR1*: *Hordeum vulgare* RAR1 (gbAAF18432); *TaRAR1*: *Triticum aestivum* RAR1 (gbABN13684); *GmRAR1*: *Glycine max* RAR1 (gbACI31548); *MdRAR1*: *Malus domestica* RAR1 (ref XP008376437); *FvRAR1*: *Fragaria vesca subsp.vesca* RAR1 (ref XP004308155); *PmRAR1*: *Prunus mume* RAR1 (ref XP008234345); *VvRAR1*: *Vitis vinifera* RAR1 (ref XP002269178); *SiRAR1*: *Setaria italic* (ref XP001267761); *OsRAR1*: *Oryza sativa* RAR1 (gb BAD27802); *CsRAR1*: *Cucumis sativus* (ref XP004159991).

3.3. Tissues Expression of *MhRAR1* in *M. hupehensis*

QRT-PCR was performed to measure the *MhRAR1* gene expression in different tissues of *M. hupehensis*. The dissociation curves were analyzed to determine the amplification specificity for *MhRAR1* and *Mhtubulin*. Only one peak existing in the dissociation curves for both *MhRAR1* and *Mhtubulin* gene, indicating that the amplifications were specific. The results showed that the mRNA transcripts of *MhRAR1* could be detected in all tested tissues and that expression was higher in leaves than that in stems and roots (Fig.4)

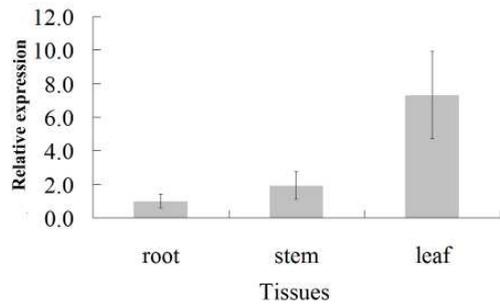


Fig. 4. Expression patterns of *MhRAR1* in different tissues using qRT-PCR in *M. hupehensis*. *MhTubulin* transcript levels were used to normalise the samples. The mean value and standard deviation were obtained from three independent experiments.

3.4. Expression of the *MhRAR1* Gene Induced by SA, MeJA and ACC in Leaves, Stems and Roots of *M. hupehensis*

In order to find out whether exogenous application of these stimuli could induce the expression of *MhRAR1* gene, *M. hupehensis* leaves were treated by plant hormones like as SA, MeJA and ACC. The expressions of *MhRAR1* gene in different organs of *M. hupehensis* were performed using qRT-PCR (Fig 5). SA, MeJA and ACC could induce the expression of *MhRAR1* gene in stems (Fig 5D, E and F) and roots (Fig 5G, H and I), but not in leaves (Fig 5A, B and C).

The amount of height of the expression level varied substantially with both time and tissue. In leaves, the transcript levels had not obviously changed upon treatment with SA (Fig 5A), MeJA (Fig 5B) and ACC (Fig 5C) during the first 48h. In stems after 4, 12 and 48 h, the transcript levels rose 2.03x, 2.64x and 2.75x, respectively, upon treatment with SA, relative to the control treatment (Fig 5D). When stems were treated with MeJA, transcript levels of *MhRAR1* rose 0.86x, 2.54x and 1.87x after the same time periods, respectively (Fig 5E). Increases of 0.88x, 1.50x and 1.65x were observed in stems following ACC treatment after 4, 12 and 48 h (Fig 5F). In roots, when compared to measurements at time 0 of the experiment, the accumulation of *MhRAR1* gene transcripts was 5.43x, 2.29x and 2.08x with SA (Fig 5G); 2.51x, 2.93x and 2.53x with MeJA (Fig 5H); 4.23x, 8.42x and 2.02x with ACC (Fig 5I), 4, 12 and 48 h post-treatment, respectively.

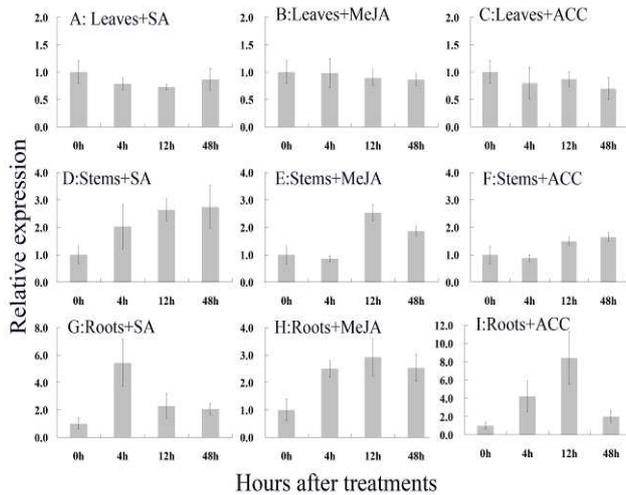


Fig. 5. Expression patterns of the *MhRAR1* using qRT-PCR in *M. hupehensis* upon treatment with SA (A, D and G), MeJA (B, E and H) and ACC (C, F and I) in leaves (A, B and C), stems (D, E and F) and roots (G, H and I), respectively. *MhTubulin* transcript levels were used to normalise the samples. Mean values and standard deviations were obtained from three independent experiments.

3.5. Induction of the *MhRAR1* Gene with the Fungus *B. Berengeriana*

In this study, we analysed the change in the expression levels of the *MhRAR1* gene in *M. hupehensis* post-infection with apple ring spot pathogen and showed that this pathogen could weakly induce the expression of *MhRAR1* (Fig 6). Levels rose at 6 hours post-inoculation (hpi), remained at the highest level at 12 hpi, and then decreased (Fig 6).

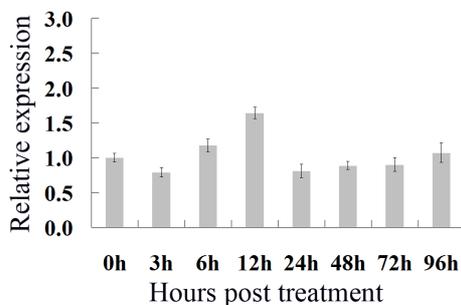


Fig. 6. Expression patterns of *MhRAR1* using qRT-PCR in *M. hupehensis* after inoculation with apple ring spot pathogen (*Botryosphaeria berengeriana* de Not. f. sp. *Parabola Nise Koganezawa et Sakuma*). Mean values and standard deviations were obtained from three independent experiments.

3.6. Subcellular Localisation of *MhRAR1* Protein

To study the subcellular distribution of the *MhRAR1* protein, the coding region of *MhRAR1* without the stop codon was fused to EGFP under the control of the CaMV35S promoter (D35S::MhRAR1-GFP) in the experiment (Fig. 7a). This plasmid, or a control (D35S::GFP), was transformed into onion epidermal cells by the particle bombardment method. It could be observed that the control proteins were uniformly distributed in cells (Fig. 7b, B1 to B3), while the *MhRAR1*-EGFP fusion protein was located in the cell wall

and cell membrane (Fig. 7b, A1 to A3).

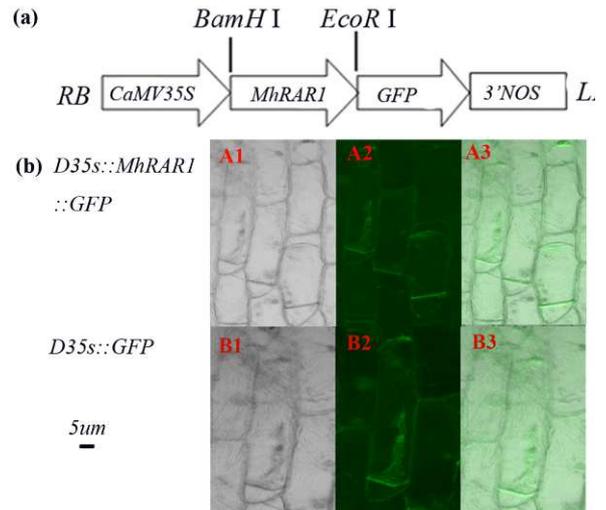


Fig. 7. The (a) *MhRAR1* fusion GFP expression vector and (b) subcellular localisation of *MhRAR1* in onion epidermal cells. Onion epidermal cells were transformed with D35S::MhRAR1-EGFP (A1 to A3) or the control plasmid D35S::EGFP (B1 to B3) using the particle bombardment method. The expression and subcellular distribution of proteins were examined under a light microscope (A1, B1) or fluorescent microscope (A2, B2) and then merged (A3, B3). Data shown are representative of three independent experiments. EGFP: enhanced green fluorescent protein.

4. Discussion

RAR1 protein contains two highly similar zinc-binding domains named CHORD-I and CHORD-II that are conserved in eukaryotic phyla except yeast [26]. There are six conserved Cys and three conserved His in CHORD-containing proteins [6]. The tandem organization of CHORD domains and conserved primary structure suggested that these proteins supply important cellular functions. In addition, plant RAR1 proteins have a 20-amino acid motif with three conserved Cys residues and a His residue in CCCH domain which was between CHORD domains I and II [4]. A cDNA sequence named *MhRAR1* gene was isolated from the woody plant *M. hupehensis* in this paper. Analysis of RAR1 protein sequence showed that *MhRAR1* protein has high sequence similarity with other herbaceous plants, such as *A. thaliana*, *N. tabacum*, *T. aestivum* and so on. The same as these plants, *M. hupehensis* *MhRAR1* protein also has two 60 aa long repeat motifs named CHORD domains at the N and C terminus, comprising about 50% of the predicted full length protein. Comparison of deduced protein sequence of *M. hupehensis* RAR1 gene with *R. communis*, *A. thaliana*, *G. max*, *N. tabacum*, *H. vulgare*, *T. aestivum* and *Saccharum* hybrid cultivar showed the same tandem organization of the 60 aa motif as well as similar spacing in each species. And *MhRAR1* contains conserved strings of invariant Cys and His residues within the CHORD domains. In addition, *MhRAR1* protein possesses a CCCH domain which also has three conserved Cys and one His between the CHORD domains. As a result, we suggested that *MhRAR1* protein may be shared the similar function with the *A. thaliana* AtRAR1 and *H. vulgare*

HvRAR1, and is an important component of R gene-mediated disease resistance.

In this report, *M. hupehensis* leaves were treated by plant hormones like as SA, MeJA and ACC to understand whether exogenous application of these stimuli could induce the expression of *MhRAR1* gene. The results showed that SA, MeJA and ACC could induce the expression of MhRAR1 gene in stem (Fig 5D, E and F) and root (Fig 5G, H and I), but not in leaf (Fig 5A, B and C), suggesting that the expression pattern of *MhRAR1* gene in various tissues under the treatment with SA, MeJA or ACC were different.

Plant innate immune responses were triggered by R genes upon pathogen attack. RAR1, which was engaged by various R genes, is an early union point in a signaling pathway [11]. In plants, RAR1 interacts directly with SGT1 (for suppressor of the G2 allele of *skp1*) and HSP90 [2,8,12,27,28]. Cytosolic HSP90 contains three distinct domains: (1) an N-terminal ATPase domain (N); (2) a substrate binding domain in the middle; (3) the C-terminal end (C) containing a dimerization domain and a MEEVD motif that binds tetratricopeptide repeat (TPR) domains of many cochaperones [8]. Takahashi et al [8] indicated that The CHORD-I domain of RAR1 interacts directly with the N terminus of HSP90. RAR1 physically interacts with SGT1 (suppressor of the G2 allele of *SKP1*) through the C-terminal CHORD-II of RAR1 and the central CS domain of SGT1 [2,29,30]. Azevedo et al [2] state clearly that the interactions among RAR1, SGT1 (suppressor of the G2 allele of *SKP1*), SCF (*Skp1-CullinDF-box*), and signalosome subunits indicate a link between disease resistance and ubiquitination. In this study, the result of subcellular localisation showed that MhRAR1-EGFP fusion protein was located in the cell wall, cell membrane and cytoplasm, suggesting that MhRAR1 protein could interact with SGT1 and HSP90 in cytoplasm. In future, we will isolate the sequences of SGT1 and HSP90 from *M. hupehensis* to study whether SGT1 and HSP90 act with RAR1.

In plants, a major form of resistance to disease caused by microbial pathogens is by expression of complementary gene pairs in the plant and pathogen, known respectively as resistance (*R*) and avirulence (*avr*) genes [31]. Direct or indirect interaction of their products activates cellular defenses that prevent pathogen colonization of the plant [32]. The most plant *R* gene class encodes predicted cytosolic proteins with a central nucleotide binding (NB) domain and C-terminal Leu-rich repeats (LRRs) [31]. NB-LRR proteins fall into two subclasses based on their different N-terminal motifs. One group possesses an N-terminal coiled-coil (CC) domain. The second group has N-terminal similarity to the cytoplasmic Toll Interleukin-1 Receptor (TIR) domains of human and *Drosophila* Toll-like receptors [31]. Muskett et al [4] reported that RAR is required by TIR- and CC-NB-LRR R genes. Barley *RAR1* is a necessary component of *R* gene-mediated resistance to the powdery mildew fungus [32]. In this study, the expression of *MhRAR1* gene was induced by the apple ring spot pathogenesis. We conjecture that MhRAR1 maybe a necessary component of R gene-mediated resistance to the apple ring spot pathogenesis.

5. Conclusion

In this study, a novel RAR1 gene *MhRAR1* was cloned from *M. hupehensis*. The results showed that MhRAR1 protein contains two CHORD domains and one plant-specific CCCH domain. In addition, the MhRAR1 contains conserved strings of invariant cysteine and histidine residues within the CHORD domains and CCCH domain. These results suggested that MhRAR1 protein from *M. hupehensis* might share the similar function with the *Arabidopsis thaliana* RAR1 and *H. vulgare* RAR1, and is an important component of R gene-mediated disease resistance. Phylogenetic analysis revealed that MhRAR1 was closely related to *R. communis* RAR1. The expression of *MhRAR1* gene was higher in leaves than that in stems and roots. SA, MeJA and ACC treatment induced *MhRAR1* expression in stems and roots, but not in leaves. Expression of *MhRAR1* was weakly induced in *M. hupehensis* after infection with *Botryosphaeria berengeriana*. In future studies we will concentrate on introducing the *MhRAR1* gene into the 'Fuji' apple variety to determine if this can enhance resistance to apple plant pathogens, such as apple ring spot, apple powdery mildew and apple canker pathogens.

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