

Curcuma longa Mitogen-Activated Protein Kinase 6 (CIMP6) Stimulates the Defense Response Pathway and Enhances the Resistance to Necrotrophic Fungal Infection

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Abstract Mitogen-activated protein kinase (MAPK) cascades are highly conserved signaling modules that transduce the externally perceived signals and play crucial role in plant defense against pathogen attack. In the present study, a turmeric (*Curcuma longa* L.) complementary DNA (cDNA) encoding a MAPK gene responsive to *Pythium aphanidermatum* infection was isolated using rapid amplification of cDNA ends (RACE)-PCR. It was designated as CIMP6 based on its high homology with *Arabidopsis AtMPK6*. The full-length cDNA of 1484 bp length carried an open reading frame (ORF) of 1176 bp encoding a 391 amino acid polypeptide. CIMP6 protein contains Thr-Glu-Tyr (TEY) motif on its activation loop with a common docking (CD) domain at the C-terminal end and belong to subgroup A of MAPK family. Southern hybridization revealed single copy of CIMP6 in turmeric genome, and its intron-exon composition showed highly conserved nature of these signaling kinases across different species. Quantitative RT-PCR showed high expression of CIMP6 in rhizome tissues of mature turmeric plants. Analysis of temporal expression revealed significant induction of CIMP6 transcript in response to defense signaling molecules and pathogen attack

at the early stages. Ectopic overexpression of CIMP6 in *Arabidopsis* plants resulted in enhanced resistance to *Botrytis cinerea* and constitutively high expression of defense responsive genes like *PDF1.2*, *PAD3*, *AOS*, *ACS2*, *ACS6*, etc. Our results suggest that CIMP6 substantiate the characteristics of AtMPK6 orthologs in defense against necrotrophic infection in plants.

Keywords MAPK · Turmeric · Transgenic *Arabidopsis* · Necrotrophs · Defense signaling

Introduction

Plants being sessile are constantly threatened by potential pathogens in the air and in the soil. To combat such pressure, plants have evolved an array of adaptive signal transduction mechanisms to perceive the external stimuli and induce intracellular responses through a series of biochemical and physiological changes (Ning et al. 2010). The activation of mitogen-activated protein kinase (MAPK) is one of the earliest signaling events in plants in response to invading pathogens (Tena et al. 2011). Plant MAPKs are similar to extracellular signal regulated kinases (ERKs) of mammals which constitute one of the most conserved transduction mechanisms in all eukaryotes (Widmann et al. 1999). The MAPK activation is carried out by their upstream kinases, MAP kinase kinases (MEKs) through phosphorylation of a threonine and a tyrosine residue in the TxY activation motif. MEKs are in turn regulated by their upstream kinases, MAP kinase kinase kinases (MEKKs) through phosphorylation of two Ser/Thr residues in the conserved Ser/Thr-X₃₋₅-Ser/Thr motif of MEK activation loop. The MAPK cascade function downstream of the sensor/receptors and transduce the signal generated into cellular responses by targeting the nuclear and cytosolic proteins

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thereby controlling the expression of downstream genes (Zhang 2008). Plant MAPKs are broadly classified into four groups (A–D). Groups A, B, and C are characterized by the presence of a Thr-Glu-Tyr (TEY) activation motif, similar to the mammalian ERK subfamily of MAPKs while group D MAPKs possesses a Thr-Asp-Tyr (TDY) activation motif, which is a unique feature of plant MAPKs (Ichimura et al. 2002; Hamel et al. 2006).

MAP kinases have been implicated in signal transduction for a multitude of stress responses in a wide variety of plants (Colcombet and Hirt 2008; Suarez-Rodriguez et al. 2010; Taj et al. 2010). *Arabidopsis thaliana* mitogen-activated protein kinase 3 and 6 (*AtMPK3/AtMPK6*) and their orthologs from other plants species belong to group A MAPK family and are involved in plant response to both biotic and abiotic stresses including wounding, response to pathogen/microbe associated molecular patterns, response to herbivory, salt and cold stress, heat stress, drought stress, ozone exposure, and heavy metal stress (Taj et al. 2010; Sinha et al. 2011). In addition, group A MAPKs are also involved in plant growth and development, pollen maturation (Voronin et al. 2004), stomatal development (Bergmann et al. 2004), embryo development (Bush and Krysan 2007), and seed formation (López-Bucio et al. 2013).

The regulation of defense responses against fungal and bacterial phytopathogens by *AtMPK3/AtMPK6* has been well documented (Asai et al. 2002). The flagellin-derived flg22 peptide causes rapid activation of *AtMPK3/AtMPK6* in *Arabidopsis* subsequently initiating pathogen defense (Asai et al. 2002). *AtMPK3/AtMPK6* also regulates the transcriptional activation of multiple genes encoding enzymes in the tryptophan (Trp) biosynthetic pathway after pathogen infection thereby resulting in the synthesis of camalexin (Ren et al. 2008). Camalexin is an important defense responsive phytoalexin which is critical for resistance against necrotrophs in *Arabidopsis* (Ren et al. 2008). Knockout mutants *mpk3* and *mpk6* are unable to express the camalexin biosynthetic genes and thus are highly susceptible to *Botrytis cinerea* infection (Ren et al. 2008). *Oryza sativa* benzothiadiazole induced MAPK1 (OsBIMK), an orthologs of *AtMPK3* in rice is involved in the regulation of defense response against *Magnaporthe grisea* (Song and Goodman 2002). *AtMPK6* also contributes to non host resistance against *M. grisea* in *Arabidopsis* (Okawa and Ishikawa 2013). The *Nicotiana tabacum* salicylic-induced protein kinase (NtSIPK, MPK6 ortholog) and wound-induced protein kinase (NtWIPK, MPK3 ortholog) are activated by various pathogens including the necrotrophs (Yang et al. 2001; Zhang and Liu 2001). Expression of a constitutively active mutant of *N. tabacum* MAP kinase kinase 2 (NtMEK2), the upstream MEK of the cascade, causes rapid activation of SIPK/WIPK thereby inducing hypersensitive response (HR) like cell death in tobacco (Yang et al. 2001). Besides, NtMEK2/SIPK/WIPK cascade also regulates the expression of genes associated with

phytoalexin and salicylic acid (SA) biosynthesis pathways in response to various pathogens or pathogen-derived elicitors suggesting the involvement of MAPK-regulated pathways against a wide diversity of different pathogens (Zhang and Liu 2001). While *AtMPK3/AtMPK6* and SIPK/WIPK have been found to be positive regulators, *AtMPK4/AtMKK2* cascade impairs resistance against necrotrophs (Brader et al. 2007). However, Wang et al. (2009) isolated BnMPK4 from *Brassica napus*, which positively regulates jasmonate (JA)-mediated defense response and play important role in resistance to *Sclerotinia sclerotiorum* and *B. cinerea* in oil seed rape. Together, these reports suggest that the regulation of MAPKs involves a highly complex network and exhibit multiple roles toward disease resistance response against phytopathogenic fungi.

Turmeric (*Curcuma longa* L.) belongs to family Zingiberaceae and is a plant of great medicinal and economic significance. However, it is highly susceptible to a necrotrophic oomycete *Pythium aphanidermatum*, which amounts to 60 % yield losses (Selvan et al. 2002). No resistant cultivars have been reported till date, and only a few lines with partial resistance are available for cultivation (Ravindran et al. 2007). Moreover, a vegetative mode of propagation together with high stigmatic incompatibility of the extant crop prevents setting up of conventional breeding approach leading to the loss of valuable resistance gene pool (Dhamayanthi et al. 2003). Currently practiced application procedures for controlling pathogen with fungicides is often ineffective due to lack of suitable forecasting methods for fungicide application. Engineering of disease resistance could be an option as has been attempted in many important crop plants (Wally and Punja 2010). However, only a very little genomic information is available to understand the molecular mechanism of stress response which restricts the engineering of resistance development in *C. longa*. In one of our previous study, we isolated four MAP kinases from turmeric with significant role in abiotic stress tolerance (Nanda et al. 2014). In the present study, we isolated a turmeric complementary DNA (cDNA) clone encoding an MAPK, *C. longa* MPK6 (*CIMPK6*) responsive to *P. aphanidermatum*, a necrotrophic oomycete causing rhizome rot disease in turmeric. We also studied the functional characteristics of *CIMPK6* and its potential role in defense response to necrotrophic infection through ectopic overexpression in *A. thaliana*.

Materials and Methods

Plant Materials and Treatments

A *C. longa* accession (Accn no.CI210-27) highly resistant to *P. aphanidermatum* was used for isolation of the MAPK gene. Rhizome harvested from mature plants were germinated in

soil-manure mix and maintained in a growth chamber with 28 °C day/18 °C night, 14 h photoperiod and 80 % relative humidity for 12 weeks for the generation of pseudostem. Super virulent field isolate of *P. aphanidermatum* (CBT-201) was obtained from Indian Institute of Spices Research (IISR), Calicut, Kerala, India and was used for inoculation. Mycelial plugs of *P. aphanidermatum* from 2-day-old potato dextrose agar (PDA) cultures were transferred to 50 ml sterile distilled water and incubated for 48 h to develop zoospores. Zoospore suspension having a concentration of 4×10^6 spores/ml was used to inoculate the plants by pinpricking the pseudostems and pouring 500 µl of the suspension upon the poked region (Kavitha and Thomas 2008). For hormonal treatment, 12-week-old turmeric plants were treated with 100 µM abscisic acid (ABA), 100 µM methyl jasmonate (MeJA), 100 µM salicylic acid (SA), and 10 µM gibberellic acid (GA). For hydrogen peroxide (H₂O₂) treatment, plants were sprayed with 10 mM H₂O₂ in sterile water. Turmeric plants treated with 0.1 % (v/v) ethanol solution served as control.

DNA and RNA Isolation

The total genomic DNA was isolated by following the protocol illustrated by Doyle and Doyle (1990) with necessary modifications. Total RNA was extracted using TRI reagent (Sigma-Aldrich, USA) and treated with DNase I (Promega, Madison, WI) as per manufacturer's instructions. The quality and concentration of isolated DNA and RNA was determined on an ethidium bromide stained agarose gel of 1.0 % (m/v). Additionally, total RNA quantity and purity was assayed using the NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Waltham, USA). RNA samples with 260/280 nm ratio between 2.0 to 2.1 were used for further analysis.

RT-PCR and Amplification of Partial cDNA

Reverse transcription was performed using the *GoScript* reverse transcription system (Promega, Madison, WI). First-strand cDNA synthesis was carried out in a 20-µl reaction mixture consisting of 2 µg of total RNA, 500 ng of 15 mer Oligo-dT primer, 2 µl of 10X RT-buffer, 2 µl of 10 mM dNTP, and 200 units of *MMuLV* reverse transcriptase (Promega, Madison, WI). The reaction was set at 25 °C for 5 min and 42 °C for 30 min followed by 5 min at 75 °C for heat inactivation. The first-strand cDNA was diluted tenfold and used as template for PCR using degenerative primers CIF-1 and CIR-1 (Supplementary Table S1). PCR was performed in a Veriti thermal Cycler (Life Technologies, Burlington, ON, Canada) using 200 µM dNTP mix, 5 pmol each of forward and reverse primer, 1X PCR buffer, and 1 unit of *Taq* polymerase (Promega, Madison, WI). PCR was performed using a gradient program with initial denaturation of 94 °C for 5 min followed by 36 cycles of 94 °C for 1 min (denaturation),

annealing at 52 °C /54 °C /56 °C /58 °C for 1 min, extension at 72 °C for 1 min with a final extension at 72 °C for 10 min. Turmeric *actin1* gene was used as the positive control, and RNA instead of cDNA was used as template for negative control in RT-PCR reaction. The PCR-amplified products were electrophoresced, gel-purified (Wizard SV gel and PCR cleanup system, Promega, Madison, WI), and cloned into pTZ57R/T vector using InsTA clone T/A cloning kit (Fermentas Life Sciences, Hannover, Germany).

DNA Sequencing and Sequence Characterization

The recombinant plasmids were purified by using Wizard Plus Minipreps DNA Purification System (Promega, Madison, WI) and sequenced using Big Dye Terminator Cycle DNA Sequencing kit (PerkinElmer, Norwalk, USA) on a 3730 DNA analyzer (Life Technologies, Burlington, ON, Canada). Sequence similarity searches were performed using BLASTn and BLASTp (<http://www.ncbi.nlm.nih.gov>). The cDNA sequence was translated using ExPaSy translate tool (<http://www.expasy.org/translate/>). Multiple sequence alignment was performed using CLUSTALX (<http://www.ebi.ac.uk>). Phylogenetic analyses were performed by neighbor-joining (*NJ*) method with 1000 bootstrapping using Molecular Evolutionary Genetic Analysis (MEGA v 6) package (Tamura et al. 2013). Motif structures of the predicted MAPK were analyzed using Multiple Expectation Maximization for motif Elicitation (MEME) tool (Bailey et al. 2006). Reverse complementation and protein structure formatting were done using ExPaSy genomics and proteomics resource tools from SIB Bioinformatics resource portal (<http://www.expasy.org>).

3' and 5' Rapid Amplification of cDNA Ends

For the isolation of full-length cDNA clones, 5'- and 3'-rapid amplification of cDNA ends (RACE)-PCR was performed using the 5'/3' RACE-PCR kit (Life Technologies, Burlington, ON, Canada) following the manufacturer's instructions. For 5'RACE, first-strand cDNA was synthesized in a 20-µl reaction mixture consisting of 4 µg of total RNA, 10X RT-buffer, 10 mM dNTP mix, 10 µM gene-specific primer, and 1 unit of reverse transcriptase at 55 °C for 60 min followed by 85 °C for 5 min. Twenty-fold diluted cDNA was used as template to amplify the 5' region using 5' RACE Abridged anchor primer (Table S1) and gene-specific primer 5MPK1 in a 50-µl reaction with 1.5 mM MgCl₂, 200 µM dNTP mix, 1X PCR buffer, and 3 units of *Taq* DNA polymerase (Fermentas, Hannover, Germany). The PCR product obtained was diluted and used as template in nested PCR using 5' MPK2 and 5' RACE Abridged Universal Amplification Primer (Table S1) following similar cycling conditions as previous. The cDNA synthesis for 3' RACE was done in same manner as in 5' RACE except that the reverse primer was replaced with a 3' RACE

Adaptor Oligo-dT primer (Table S1). Diluted cDNA was used as a template for PCR amplification of 3' region using a gene-specific primer 3' MPK1 and 3' RACE Abridged universal amplification primer (Table S1). PCR amplification was programmed with following parameters: 94 °C for 3 min followed by 35 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min 30 s with a final extension of 10 min at 72 °C.

Amplified fragments were cloned and sequenced as discussed previously. The full-length cDNA of *CIMPK6* was obtained by aligning the RACE amplified sequences with the cloned partial cDNA fragment. Full-length cDNA including the 5'- and 3'-untranslated regions (UTRs) were amplified using gene-specific primers CIMPK6-1F and CIMPK6-1R. The PCR reaction consisted of a 50- μ l reaction mixture including 200 μ M dNTP mix, 1XPCR buffer, and 3 units of *Taq* DNA polymerase and 100 ng cDNA as template. The reaction conditions were 94 °C for 3 min followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 2 min. Gene-specific primers CIMPK6-1F and CIMPK6-1R were also used to amplify the genomic clone of CIMPK6 using *C. longa* genomic DNA as the template. The PCR was carried out using 50 ng of genomic DNA in a 25- μ l reaction mixture consisting of 25 mM MgCl₂, 10X PCR buffer, 25 mM dNTP mix, and 1 unit *Taq* DNA polymerase. PCR cycling parameters were 94 °C for 2 min, followed by 35 cycles of denaturation at 94 °C for 45 s, 55 °C for 45 s, 72 °C for 2 min, and a final extension of 10 min at 72 °C.

Southern Blot Analysis

Ten micrograms of genomic DNA from *C. longa* (Accn. C1210-27) was independently digested with *EcoR* V, *BamH* I, and *Xba* I (Fermentas, Hannover, Germany) and electrophoresed on a 1.2 % agarose gel. The separated fragments were transferred to a Hybond-N⁺ nylon membrane (Amersham Pharmacia Biotech) using 0.5 N NaOH transfer buffer through capillary blotting. The nylon membrane was baked at 80 °C for 2 h to fix the DNA onto its surface. A 289-bp fragment was PCR amplified from the 3' end (217 bp of the 3' UTR and 72 bp of the coding region) of the *CIMPK6* gene by using primers CISM PK-F and CIMPK6-1R and used as the hybridization probe. The probe was prepared by labeling with digoxigenin (Roche Diagnostics, Switzerland) using a mixture of DIG-labeled and standard dNTPs in 1:3. The membrane was blocked with DIG Easy Hyb (Roche Diagnostics, Switzerland) for 1 h at 62 °C and hybridized with digoxigenin labeled probes at 65 °C for 15 h in a hybridization chamber. The membrane was subjected to multiple washes with 0.1 \times SSC (15 mM NaCl, 1.5 mM Na-citrate, pH 7.0) containing 0.1 % sodium dodecyl sulphate at 65 °C for 20 min and the probe hybridization was detected as per manufacturer's instructions.

CIMPK6 Expression Analysis Through Semi-Quantitative and Quantitative Real-Time PCR

Reverse transcription was performed as described above. Real-time PCR analysis of *CIMPK6* gene was performed using gene-specific primers CIMPK6-3F and CIMPK6-3R (Table S2). Specificity of the designed primers was determined by PCR amplification of the genomic DNA. For semi-quantitative PCR, amplification was performed with initial denaturation of 94 °C for 5 min followed by 36 cycles of 94 °C for 1 min (denaturation), annealing at 52–58 °C for 1 min, extension at 72 °C for 1 min with a final extension at 72 °C for 10 min. Equivalence of cDNA in different samples were verified using PCR reactions for *Actin1*. The amplified products were analyzed on 1.2 % agarose gel and visualized by staining with ethidium bromide. The quantitative real-time PCR (qRT-PCR) was performed with a total volume of 10 μ l reaction mixture containing 5 μ l of FASTSYBR Green PCR mix (Life Technologies, Burlington, ON, Canada), 1 μ l each of forward and reverse primers (5 μ M), 1 μ l template cDNA, and 1 μ l of nuclease free water. A fast qRT-PCR was carried out on a StepOne Plus real-time PCR system with temperature condition of 95 °C for 60 s (initial denaturation) followed by 40 cycles of 95 °C for 15 s, and 60 °C for 30 s. A dissociation curve analysis was done by using cycle of 65 °C for 15 s followed by a slow temperature increase to 95 °C at the rate of 0.2 °C /s. Three biological samples for each reaction and three technical replicates for each biological sample were used for qRT-PCR. Constitutively expressed housekeeping gene *actin1* from *C. longa* was used as an endogenous control. The relative expression was evaluated using the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen 2001). A two-way analysis of variance (ANOVA) and multiple comparisons using uncorrected Fischer's least significant difference (LSD) test was performed to determine the statistical significances of the results. The differences in the results were scored as statistically significant at $p < 0.05$.

Plasmid Construction and Plant Transformation

The complete open reading frame of *CIMPK6* was amplified using primers CIMPK6-ORF-F and CIMPK6-ORF-R (Table S1). The amplified product was inserted into the pENTR/D-TOPO vector to develop gateway entry clone "pENTR/D-TOPO-*CIMPK6*" using pENTR/D-TOPO cloning kit (Invitrogen) following manufacturer's instructions. The cloning of the fragment was confirmed by sequencing. The full-length cDNA of *CIMPK6* was then transferred from the entry vector and inserted downstream of Cauliflower mosaic virus (CaMV) 35S promoter in the gateway destination vector pB7WG2 (Karimi et al. 2002) using the LR clonase reaction. Floral dip method (Clough and Bent 1998) was used to transform *Arabidopsis* lines using plant expression vector

with *35S::CIMP6* construct in an *Agrobacterium tumefaciens* LBA4404 strain. Then, 50 mg/L Basta solution was sprayed once in a week for a total of 3 weeks to select transgenic *Arabidopsis* plants, which were further confirmed by PCR. The selection process was repeated for two generations to get homozygous transgenic plants.

Evaluation of Transgenic Plants for Resistance Against Fungal Infection

B. cinerea cultures were grown on PDA medium and incubated at room temperature (RT; 21 ± 2 °C) for 10 days. Conidia were collected with 500 μ l of sterile distilled water and an inoculation suspension was prepared containing 6×10^5 conidia/ml in 0.01 M glucose and 6.7 mM KH_2PO_4 (pH 5.0). Each leaf was inoculated twice by placing 5 μ l droplets of suspension on to leaf surface. Mock treatment was done using sterile distilled water. Inoculated plants were incubated at 20 ± 2 °C under dark conditions, and the symptoms were evaluated after 96 h. Lesions were classified as (1) spreading with progressive development and, (2) non-spreading lesions with droplet marks. Disease severity was calculated based on the percentage of spreading lesions.

Expression Analysis of Other Stress-Responsive Genes in Transgenic Plants

qRT-PCR was performed to monitor the expression analysis of *CIMP6* and other important defense-responsive genes in transgenic *A. thaliana*. Leaf samples were collected from WT and transgenic lines at different time points post inoculation with *B. cinerea*, frozen in liquid nitrogen, and stored at -80 °C. Total RNA isolation, cDNA synthesis, and qRT-PCR analysis were performed as discussed earlier. Gene-specific primer sequences used in this study are given in supplementary Table S2.

Results and Discussion

Isolation of Full-Length cDNA

MAP kinases are members of hierarchically arranged protein kinases that rapidly transduce extracellular signals into appropriate intracellular responses in the plants to defend against the adverse conditions (Taj et al. 2010). In our attempt to clone a *C. longa*-specific MAP kinase responsive to phytopathogenic fungi, degenerative primers were designed from the conserved regions of the MAP kinases. The conserved motif GAYGIVC from the subdomain I corresponds to the forward primer CIF-1 while the conserved motif MTEYVVT from subdomain VIII corresponds to the reverse primer CIR-1 (Hanks et al. 1988). A single cDNA fragment of 501 bp was amplified through

RT-PCR. The PCR products were cloned, and identity of the cloned product was checked through BLASTp and BLASTn search analysis confirmed high degree of similarity of amplified PCR product with known MAP kinases from other plant species. One clone with 97 % sequence similarity with AtMPK6 (AAM53295) was selected for isolation of full-length cDNA through RACE-PCR.

The gene-specific primer 3MPK1, designed from the partial cDNA sequence, was used for 3'RACE to amplify a single fragment of 1026 bp from the 3' end of cDNA of the MAPK gene (Fig. S1a). Another round of nested PCR with 3MPK2 and 3' RACE abridged universal amplification primer was used to confirm the amplification of the desired fragment. For 5' RACE, gene-specific primer 5MPK1 and 5' RACE anchor primer were employed to amplify a fragment of 646 bp (Fig. S1b) which was confirmed with nested primer 5MPK2. The PCR products from the 3' and 5' RACE were aligned together to deduce the full-length cDNA. Two gene-specific primers CIMP6-1F and CIMP6-1R were designed based on the deduced information from the RACE products and used for PCR amplification of the full-length cDNA. The PCR product of 1484 bp (Fig. S1c) was cloned, sequenced, and was found to be identical to the deduced cDNA. This suggests that RACE-PCR can be efficiently used to clone the full-length MPK genes in plants. RACE amplification has also been used earlier for the cloning and characterization of *CbMPK3* from *Chorispora bungeana* (Zhang et al. 2006), *CsNMPK* from *Cucumis sativus* (Xu et al. 2008), and four already known MAP kinases from turmeric (Nanda et al. 2014).

Sequence Analysis of CIMP6

The 1484 bp cloned cDNA included an 1176 bp open reading frame (ORF), a 91 bp 5' UTR upstream of the start codon, and a 217 bp 3' UTR downstream of the stop codon. The entire ORF of the cloned cDNA encodes a protein of 391 amino acid residues with a predicted molecular weight of 44,348.5 Da and a calculated isoelectric point (pI) of 5.55. The deduced protein sequence exhibit 97 % identity with AtMPK6 (AAM53295) and 95 % identity with *Zea mays* MPK6 (ZmMPK6, ACG37232). Similar to AtMPK6 and ZmMPK6, the predicted protein sequence contains a conserved phosphorylation activation motif (Thr-Glu-Tyr) and a C-terminal extension containing a common docking (CD) domain. Based on *Arabidopsis* MPK nomenclature suggestions (Ichimura et al. 2002), the cloned cDNA was designated as *CIMP6* and deposited in the National Centre of Biotechnology Information (NCBI) GenBank database (Accession number KP782042). SOPMA analysis revealed that CIMP6 protein constitutes major α -helices (48.08 %), random coils (33.25 %), extended strands (12.28 %), and β -turns (6.39 %) (Fig. S2a). Hydropathy analysis using vector NTI

suite 10 showed strong and maximum peaks toward the negative side suggesting that CIMP6 is a hydrophobic protein (Fig. S2b).

Based on genetic distance among the protein sequences, the phylogenetic tree between CIMP6 and known MAPKs from other plants showed clustering of the predicted protein sequence within group A of the MPK family (Fig. 1a). Besides highest similarity with AtMPK6 and ZmMPK6, CIMP6 protein also exhibited 95 % identity to BnMPK6 (ADM89008) from *B. napus* and 94 % to OsMPK6 (ACD76439) from *O. sativa* and NtSIPK (AAB58396) from *N. tabaccum*. The other important members of group A MPK family, OsMPK3 (ABH01189), AtMPK3 (NP_190150), and NtWIPK (BAA09600), shared 81 % similarity with CIMP6 protein. Phylogenetic relationship was further confirmed by multiple sequence alignment of CIMP6 with conserved MAP kinases spanning approximately about 300 amino acids. Sequence alignment using ClustalX revealed 11 highly conserved protein kinase subdomains in CIMP6 that characterize the MAPKs (Fig. 2). Conserved motif analysis using Multiple Expectation Maximization for Motif Elicitation (MEME) identified 18 conserved motifs from CIMP6, 11 of which perfectly corresponded with I to XI subdomain found in various serine/threonine protein kinases (Fig. 1b). Motif I and motif II corresponded with subdomain VIII (DFMTEYVV/FTRWRAPE) and subdomain IX (YTAAIDVWSVGCJFM/A) and exhibited highest conservation with other known MPK6 proteins (Fig. 1c). Motif XIII corresponded with the CD domain in the extended C-terminal region with the

consensus sequence HPYLA/NSLHDISDEPVC (Fig. 1d) which functions as the binding site for MAP kinase kinase (M2K). The above sequence analysis suggest that CIMP6 is highly similar to MPK6 proteins from other plant species and possibly exhibit the same function that of its orthologs.

Genomic Organization of CIMP6

To determine the genetic representation of *CIMP6* gene in turmeric genome, we deduced the copy number of the gene using southern hybridization. Briefly, *C. longa* genomic DNA was digested independently with three restriction endonucleases, *EcoR V*, *BamH I*, and *Xba I* and hybridized with a 289 bp digoxigenin-labeled probe designed from the 3' UTR region of the *CIMP6*. DNA digested with all the three restriction enzymes detected only a single hybridization signal (Fig. 3a). The hybridization pattern suggested that *CIMP6* is represented by a single copy locus in the turmeric genome.

To get insights into the gene structure of *CIMP6*, a 2345-bp genomic DNA clone was amplified using turmeric genomic DNA as template and gene-specific primers CIMP6-1F and CIMP6-1R corresponding to the 3' and 5' termini of the *CIMP6* cDNA. Alignment of the genomic structure of *CIMP6* gene and the corresponding cDNA revealed the presence of six exons and five introns within the coding region of *CIMP6* (Fig. 3b). The size of the exons varied from 130 bp (exon II) to 333 bp (exon IV), while size of the introns varied from 93 bp (Intron IV) to 489 bp (Intron II). All the introns were found to be A + T rich (AT content ranged from

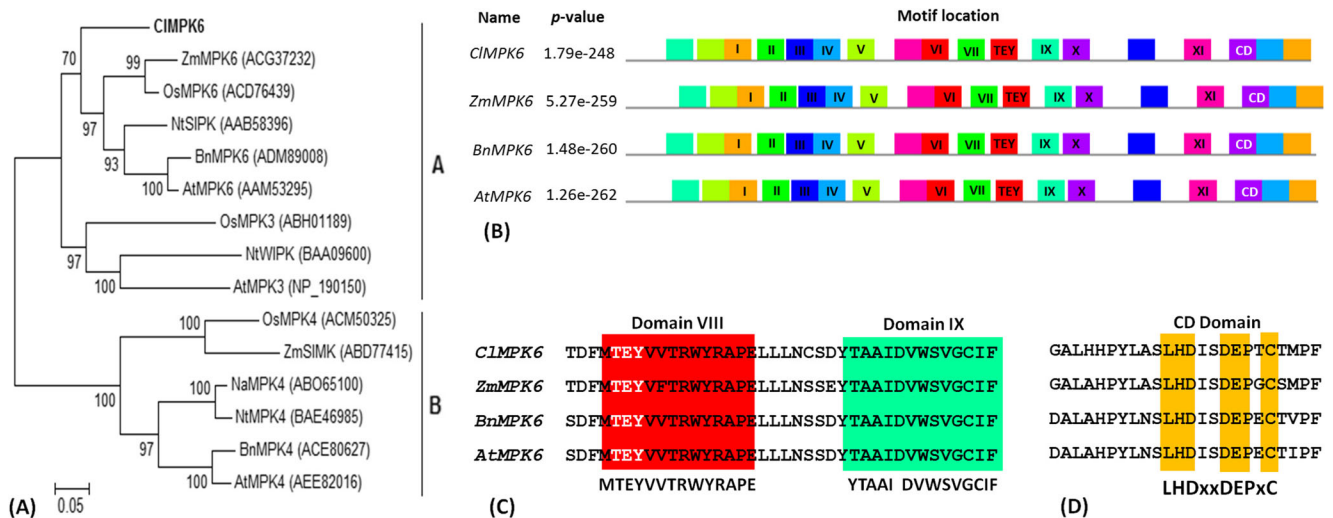


Fig. 1 Phylogenetic and domain analysis of CIMP6. **a** Phylogenetic tree of CIMP6 and MAPKs from other plant species. The tree is constructed with the MEGA 6.0 program. Numbers on the branches indicate the percentage of bootstrap replications. The numbers against the MAPKs indicates accession numbers as listed in the GenBank database. **b** Schematic diagram of conserved amino acid motifs of CIMP6 and MPK6 orthologs as analyzed through MEME 4.0 program. The black solid line represents corresponding MPK6 and its

length while the colored boxes represent different motifs and their position in each sequence. The 11 characteristic conserved motifs of MAPKs are named with roman numerical. **c** Amino acid sequence alignment of CIMP6 and its orthologs at the highly conserved domain VIII and domain IX. The signature amino acid sequence of domain VIII and IX are highlighted in red and green color, respectively. **d** Comparison of CD domain among CIMP6 and its orthologs. Conserved amino acid sequence of the common docking site is highlighted in yellow

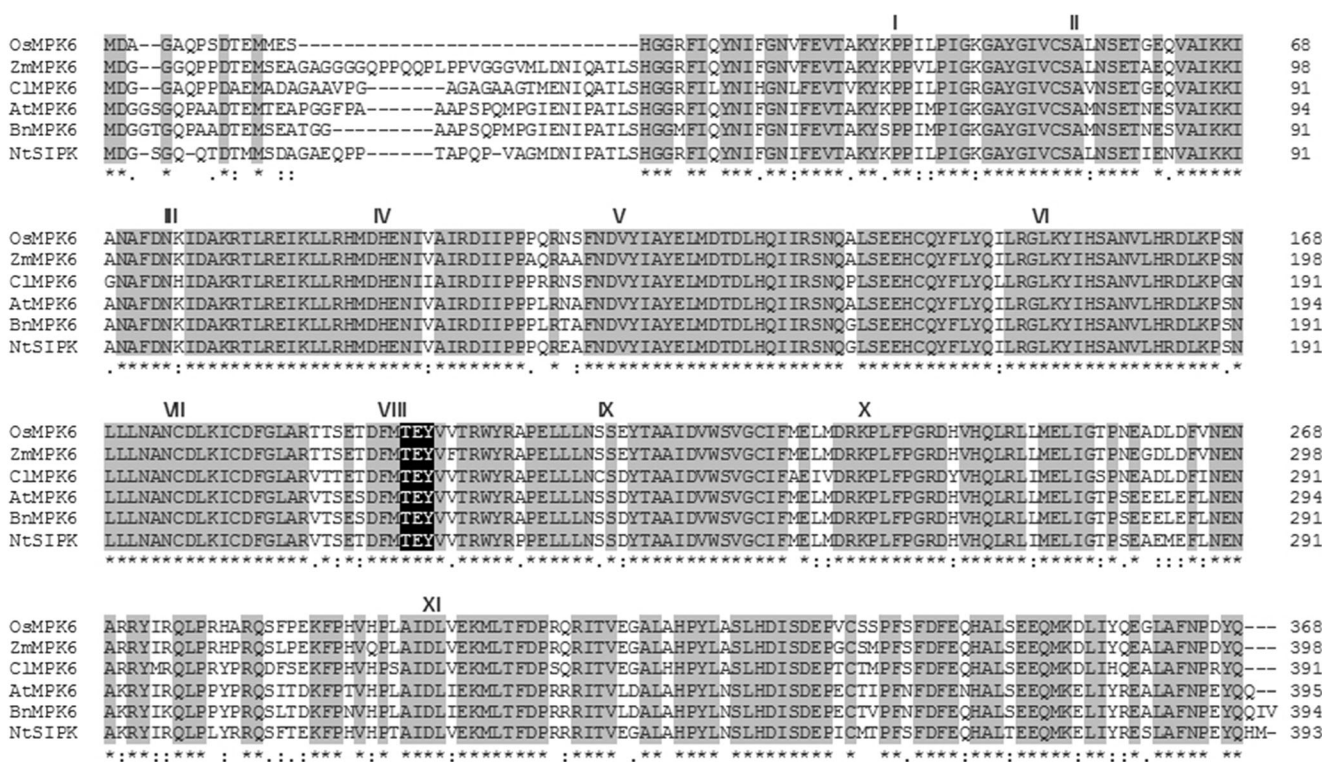


Fig. 2 Alignment of deduced amino acid sequences of CIMPK6 with MPK6 orthologs from other plant species. The 11 conserved domains of protein kinases are marked with roman numerals and shaded. Thr/x/Tyr

(TX) residues of the phosphorylation loop are highlighted in black. *Os Oryza sativa*, *Zm Zea mays*, *At Arabidopsis thaliana*, *Bn Brassica napus*, *Nt Nicotiana tabacum*

61 to 68 %) and possessed canonical consensus dinucleotide GT-AG sequence at the 5' and 3' splice junctions (Supplementary Table S3), a typical feature of many plant introns. An intron phase analysis revealed that Intron II, III, and IV were within phase 0 while intron I and V exhibited phase 2 and phase 1, respectively (Fig 3b). Comparison of CIMPK6 gene structure with orthologs from *A. thaliana* (*AtMPK6*), *Populus trichocarpa* (*PtMPK6*) and *Brachipodium*

distachyon (*BdMPK6*) indicated that both the number and size of the exons were highly conserved. Whereas all the orthologs maintained similar intron phases, the intron lengths varied among the species as has been previously reported (Nanda et al. 2014). *AtMPK6* introns were similar while *BdMPK6* and *PtMPK6* introns were much larger as compared to corresponding CIMPK6 introns. Thus, it can be suggested that MPK6 has a relatively constant and conserved intron-exon composition

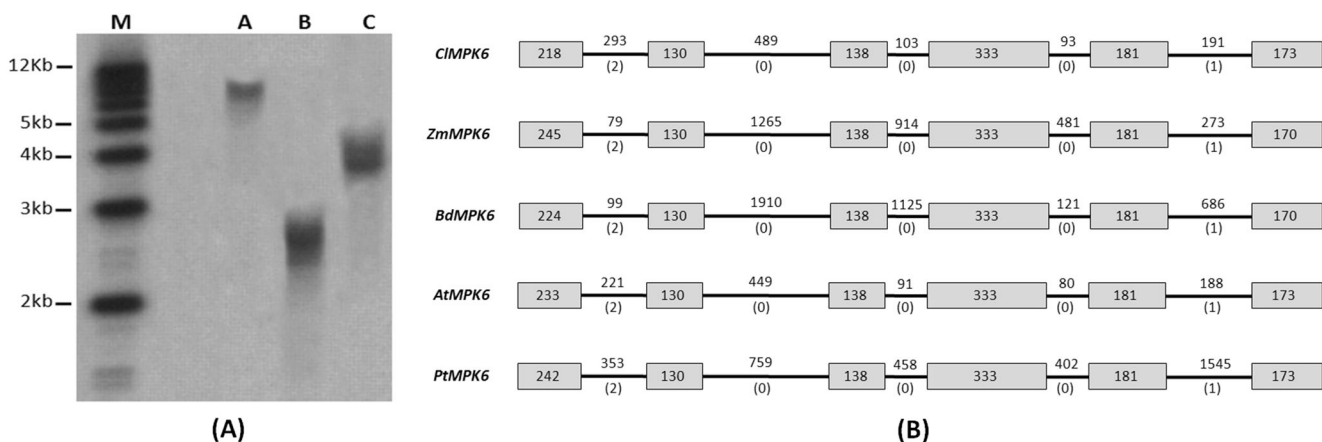


Fig. 3 Genomic and structural organization of CIMPK6. **a** Southern blot analysis of CIMPK6. *A EcoR V*, *B BamH I*, and *C Xba I*. Genomic DNA independently was digested with specific restriction enzymes, separated on a 0.8 % agarose gel, blotted onto a nylon membrane, and hybridized with CIMPK6 specific probe. **b** Comparison of CIMPK6 gene structure

and its orthologs from maize (*ZmMPK6*), *Brachipodium* (*BdMPK6*), *Arabidopsis* (*AtMPK6*), and *Populus* (*PtMPK6*). Introns are represented by dark lines and exons by boxes. Introns and exon length are given in base pairs. The bracketed numbers corresponds to intron phase

across various species and strong negative selection pressure exists for any changes in the genomic organization of this gene.

Expression Analysis of *CIMPK6* by qRT-PCR

To observe the specificity of spatial expression in *CIMPK6*, we performed semi-quantitative RT-PCR in three different organs: leaves, rhizomes, and roots at early seedling and mature stages, respectively. RT-PCR results showed that the expression of *CIMPK6* transcript was higher in rhizomes, followed by leaves and roots, albeit differentially at both the stages (Fig. 4a). In the early seedlings, the *CIMPK6* transcript level was highest in the rhizomes followed by leaves while the expression in the roots was negligible. At the mature stage, *CIMPK6* showed similar expression but the transcript levels were significantly higher in all the tissues as compared to seedling stage. These results revealed that mature turmeric plants exhibit high transcription level for *CIMPK6* in all tissue types when compared to the young plants. Similar observation has been reported for the expression of *CbMPK3* from *C. bungeana* (Zhang et al. 2006) and *AhMPK3* from *Arachis hypogea* (Kumar et al. 2009). This suggests that *CIMPK6* might be responsible for exhibiting exceptional signaling responsibilities in differentiation and growth of turmeric plants at the later developmental stage. Additionally, expression of *CIMPK6* was significantly higher in the rhizome tissues implying its involvement in the direct perception of biotic stress signal at the site of infection.

CIMPK6 orthologs from other plants species has been found to be transcriptionally regulated in response to fungal phytopathogen (Menke et al. 2004; Galletti et al. 2011; Liu et al. 2014). To determine the expression efficiency of *CIMPK6* in response to *P. aphanidermatum*, a time course expression analysis post inoculation was carried out using qRT-PCR (Fig. 4b). Upon infection, *CIMPK6* was induced sixfold within 6 h post inoculation (hpi) and remained steady by 12 hpi. *CIMPK6* transcript levels were significantly upregulated (greater than 18-fold) thereby reaching a peak at 24 hpi before gradually declining by 11.4-fold at 48 hpi and 6.13-fold at 72 hpi. Menke et al. (2004) has reported that *AtMPK6* maintain basal resistance to virulent bacterial pathogen and activate exclusive resistance against virulent as well as avirulent fungal pathogen in *Arabidopsis*. *AtMPK6* (an ortholog of *CIMPK6*) play a major role in upregulating the genes involved in camalexin biosynthesis (Ren et al. 2008) and silencing of *AtMPK6* leads to impaired resistance against *B. cinerea* (Han et al. 2010). Recently, Liu et al. (2014) reported that overexpression of soybean MPK6 in *Arabidopsis* caused induced defense response against pathogenic fungi. Hence, it could be suggested that *CIMPK6* might be a putative regulator of initial defense response against *P. aphanidermatum* infection in turmeric.

Pathogen infection often results in changes in the level of phytohormones in plants (Robert et al. 2007). Various phytohormones such as jasmonic acid (JA), SA ABA, and GA acts as major signaling molecules in regulating plant defense responses against various pathogens, pests, and abiotic stresses (Bari and Jones 2009). To determine the role of *CIMPK6* in response to potential defense signaling molecules, we studied the level of *CIMPK6* transcript following temporal exogenous

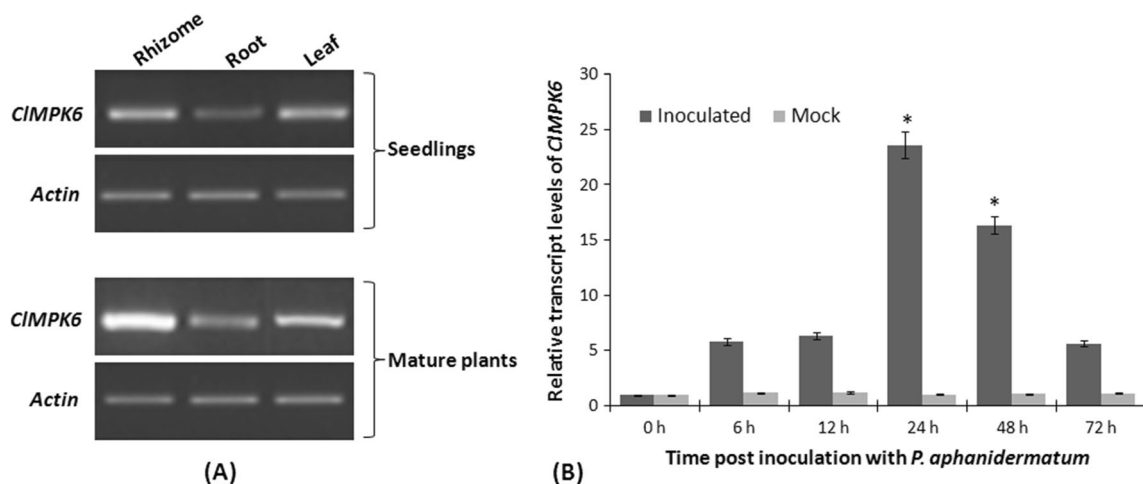


Fig. 4 Expression analysis of *CIMPK6* in turmeric. **a** Expression levels of *CIMPK6* in different tissues of *C. longa* at the seedling and mature stages using semi-quantitative RT-PCR. All RT-PCR reactions were taken from the same batch of cDNA and were repeated at least three times with identical results, of which only one image is shown here. **b** Relative expression of *CIMPK6* at different time points post-inoculation with *P.*

aphanidermatum. Expression levels are shown as relative those of the control plants (0 h), which were set to 1. Error bars show standard deviations for three independent experiments. Asterisk indicates the significant difference (at P value <0.05) between infected and mock samples identified through two-way ANOVA test

application of MeJA, ABA, SA, H₂O₂, and GA using qRT-PCR. The results showed that a basal level of *CIMPK6* is always maintained in turmeric rhizome which is induced upon prevalence of stress (Fig. 5). Physiological differences within the plant at the time of stress treatment or plant circadian rhythm could be accounted for difference in the basal transcript level post treatment with different signal molecules. MeJA, which is a major signaling molecule for necrotrophic pathogen response (Wasternack 2007), induced the accumulation of *CIMPK6* transcript (greater than 6 fold) as early as 30 min post treatment which gradually increased and reached a peak (27.5-fold) at 12 h followed by an abrupt decline (9.9-fold) at 24 h and rebound upregulation (17.3-fold) by 48 h (Fig. 5a). With ABA application, expression of *CIMPK6* was found to be rapidly upregulated (9.2-fold) within 1 h post treatment followed by a sudden decline (3.4-fold) at 6 h. At 12 h, *CIMPK6* again showed a rapid induction (12.2-fold) followed by a decline to 3.9-fold at 24 h. After this, the expression level maintained a steady level of 3.16-fold at 48 h, which is considerably higher than the basal level (Fig. 5b). Exogenous treatment with SA led to pronounced increase in *CIMPK6* transcript level (6.6-fold) within 30 min followed by sudden decline to basal level within 1 h (Fig. 5c). GA accumulation in plants is often associated with pathogen and wound stress response (Bari and Jones 2009). So, we also studied the expression of *CIMPK6* in response to GA. *CIMPK6* transcript level showed a gradual increase (6.67-fold) by 30 min, swift down-regulation by 1 h (2.3-fold), followed by steady state increase and reaching a peak (10.6-fold) by 24 h. Finally, the expression level went down to 3.2-fold at 48 h (Fig. 5d). Structural damage and pathogen

infection also causes H₂O₂ accumulation in plants (Quan et al. 2008). However, exogenous application of H₂O₂ had no significant impact on the expression pattern of *CIMPK6*. The simultaneous induction of *CIMPK6* by *P. aphanidermatum* infection as well as several signaling molecules suggests that *CIMPK6* must be acting as a prospective regulator of multiple signaling pathways leading to defense response against rhizome rot disease in turmeric.

Generation of Transgenic *Arabidopsis* Plants and Inoculation Assay

To evaluate the functional characteristics of *CIMPK6*, we constructed a plant transformation vector containing *CIMPK6* under the transcriptional control of a CaMV 35S promoter and transformed the *A. thaliana* ecotype Columbia using floral dip method. *A. thaliana* transformed lines were used for functional analysis due to complexity in transformation systems and low transformation efficiency in turmeric. After two rounds of treatment with Basta solution, 34T₁ transgenic plants grown in soil were selected based on their resistance to BASTA. To verify the presence of *bar* gene, genomic DNA was isolated and PCR was performed using primers for the marker genes, *bar* marker, and *CIMPK6*. Out of the 34 plants analyzed, 29 plants gave expected amplification of 550 and 720 bp fragments for *bar* and *CIMPK6* sequences, respectively (data not shown). Eight selected T₀ transgenic plants were confirmed by Southern hybridization. Genomic DNA digested with *SacI*, and hybridized with a *bar* gene probe displayed one or two copies of fragments in the transgenic plants while no hybridizing band was observed in the WT plant (Fig. 6a). Total RNA

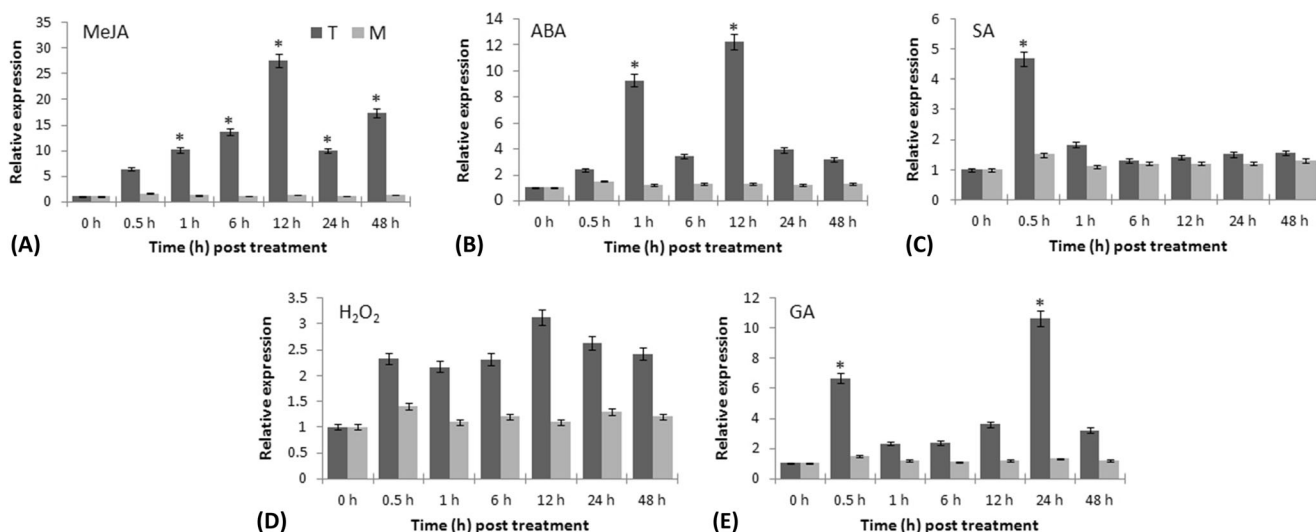


Fig. 5 Quantitative RT-PCR analyses of the expression profiles of *CIMPK6* post treatment with phytohormones and molecules involved in defense signaling. Expression levels are shown as relative those of the control plants (0 h), which were set to 1. The mean expression value was

calculated with three replications. Asterisk indicates the significant difference (at *P* value <0.05) between infected and mock samples identified through two-way ANOVA test. *MeJa* methyl jasmonate, *ABA* abscisic acid, *SA* salicylic acid, *H₂O₂* hydrogen peroxide, *GA* gibberellic acid

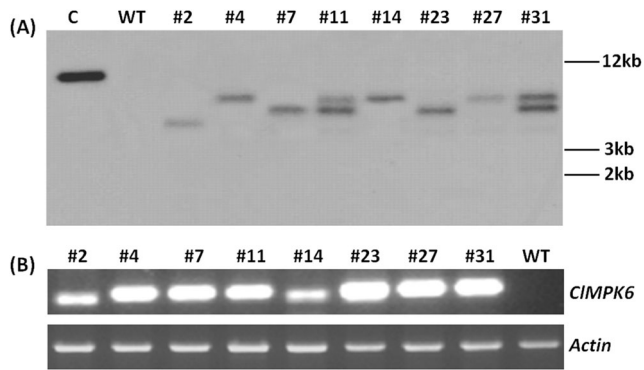


Fig. 6 Detection of transgenic *Arabidopsis* plants by Southern hybridization and semi-quantitative reverse transcription PCR. **a** Southern blot analysis of *CIMPK6* transgenic plants. *C* positive control (pB7WG2-*CIMPK6* vector), *WT* non-transformed wild type control plant, numbers 2, 4, 7, 11, 14, 23, 27, and 31 represents randomly selected transgenic *Arabidopsis* plants. **b** Semi-quantitative RT-PCR on transgenic and *WT* plants. The upper panel shows the results obtained from using *CIMPK6* specific primers while the lower panel shows the amplification of the same substrate using *Actin* primers for loading control

was isolated and semi-quantitative RT-PCR was performed to determine the expression of *CIMPK6* gene in the seven selected transgenic plants (Fig. 6b). Three putative transgenic plants #4, #23, and #31 with higher level of expression of *CIMPK6* transcript were selected for further functional characterization.

Recent evidences demonstrate the involvement of *AtMPK6*, its closest homolog *AtMPK3*, and upstream components of MAPK cascade in regulating defense response against various necrotrophic pathogens (Galletti et al. 2011; Pitzschke et al. 2009; Liu et al. 2014). To test the effect of *CIMPK6* on resistance to necrotrophs, transgenic *Arabidopsis* plants ectopically expressing *CIMPK6* were studied for their resistance against *B. cinerea*. *B. cinerea* is a necrotrophic fungus that causes grey mold diseases in a wide range of plant species including *A. thaliana*. Fully developed leaves from three selected transgenic lines and *WT* plant were detached and inoculated with a virulent strain of *B. cinerea*. After 96 h post inoculation, disease symptoms in the form of spreading lesions were observed in the *WT* plants, whereas the leaves of the *WT* plant did not show any symptoms (Fig. 7a). Close examination revealed fine bright spot of infection site in the transgenic lines but the disease did not spread as in the case of the *WT*. These results suggest that *CIMPK6* transgenic plants exhibited enhanced levels of resistance against *B. cinerea* and can act as a potential candidate gene for imparting resistance against necrotrophs.

Additionally, qRT-PCR was performed to determine the expression level of *CIMPK6* in the three transgenic plant post inoculation with *B. cinerea*. The result showed no change in the transcript level in the *WT* while the transgenic lines revealed eightfold increase in transcript level of *CIMPK6* (Fig. 7b). qRT-PCR evaluation of the orthologous gene *AtMPK6* revealed four- to fivefold increase in transcript

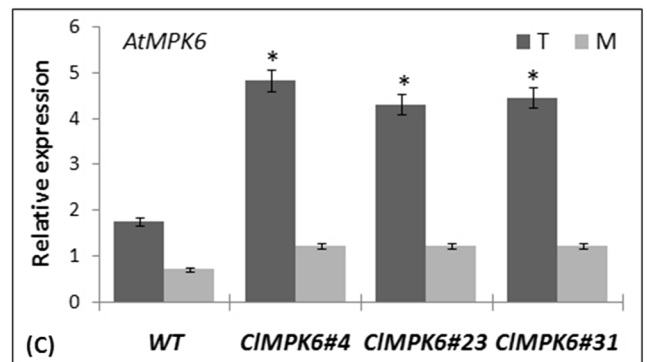
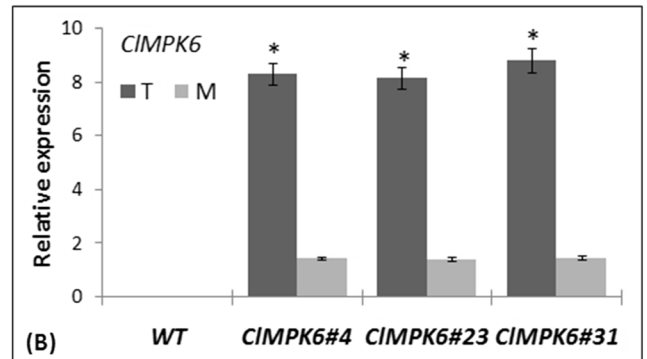
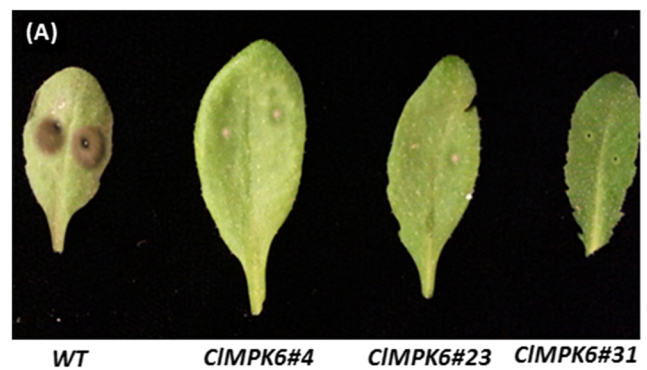


Fig. 7 Over expression of *CIMPK6* in *Arabidopsis thaliana* increases the resistance to *Botrytis cinerea*. **a** In vitro inoculation of *Botrytis cinerea* on *Arabidopsis* leaves. Leaves were infected with *B. cinerea* spores and incubated in humid conditions for symptom development. The wild type (*WT*) leaves exhibited clear disease symptoms within 4 days of inoculation, while the transgenic lines #4, #23, and #31 showed no symptomatic development. **b, c** Quantitative real-time PCR analysis of *CIMPK6* and *AtMPK6*, respectively, in the wild type (*WT*) and three selected transgenic lines #4, #23, and #31 at 96 h post infection with *B. cinerea*. Expression levels are shown as relative to those of the control plants (0 h), which were set to 1. Asterisk indicates significant difference at *P* value <0.05. The experiments were repeated three times

abundance in all the transgenic lines while the accumulation of the *AtMPK6* transcript was negligible (1.7-fold) in the *WT* (Fig. 7c). These results make us to assume that *CIMPK6* might be responsible for the induction of endogenous *AtMPK6* in the transgenic lines upon pathogen infection. Currently, our lab is involved in the development of knockout lines for *CIMPK6* which will throw more insights into its possible role in the modulation of *AtMPK6*.

Expression Analysis of Defense Responsive Genes in Transgenic Plants

Transgenic plants showing improved resistance to *B. cinerea* were analyzed for level of expression of various other defense responsive genes using qRT-PCR (Fig. 7; S3 and S4). JA mediated signaling response is one of the most important defense mechanisms exhibited against necrotrophic infection in plants (Antico et al. 2012). Hence, we examined the temporal expression of JA signaling genes post fungal infection. *Plant defensin (PDF) 1.2*, a prominent gene regulated synergistically by ET and JA showed high transcript accumulation (10–15-fold) in the transgenic plants as early as 3 h post-inoculation with gradual increase by 40–55-folds at 24 h (Fig. 8a; S3 and S4). Overexpression of *MPK6* in *Arabidopsis* causes the phosphorylation of ethylene response factor (*ERF*) 104 which in turn causes the activation of defensin genes *PDF 1.2a* and *PDF 1.2b* (Bethke et al. 2009). The expression of phytoalexin deficient 3 (*PAD3*) and *thionin 1.2 (Thi 1.2)* was significantly higher in the transgenic lines (Fig. 8b, c; S3 and S4). However, only a slight increase in transcript levels of these two genes could be seen in the WT even after 24 h after inoculation. Pathogen responsive activation of *MPK6* in *Arabidopsis* leads

to in time upregulation of multiple genes including *PAD1* and *PAD3* which encodes enzymes for camalexin biosynthesis (Ren et al. 2008). Other JA responsive gene transcripts like allene oxide synthase (AOS) and allene oxide cyclase (AOC) also showed higher expression levels in the transgenic lines compared to WT plants (Fig. 8d, e; S3, and S4). These results indicate a possible JA-dependent signaling response toward *B. cinerea* resistance in *CIMPK6* transgenic plants.

JA-mediated signaling also work in concert with ET thereby eliciting defense response (Guo and Ecker 2004). As *PDF 1.2* is also known to be regulated by ethylene (Lorenzo et al. 2003), we also studied the expression of 1-aminocyclopropane-1-carboxylic acid synthase 2 (*ACS2*) and *ACS6* involved in ET biosynthesis. Our analysis revealed that the expression of *ACS2* and *ACS6* was significantly higher in transgenic lines as compared to WT. *ACS2* was upregulated within 6 h of infection and its expression firmly rose at later infection stages (Fig. 8f). *ACS6* was significantly upregulated (22–25-folds) as early as 3 h post infection in transgenic plants. However, the expression level gradually decreased to 13–15-folds by 24 h which is still significantly higher than the WT (Fig. 8g; S3 and S4). Recent reports have shown that tobacco *SIPK/Nif4/WIPK* and *Arabidopsis MPK3/MPK6* play key role in regulating

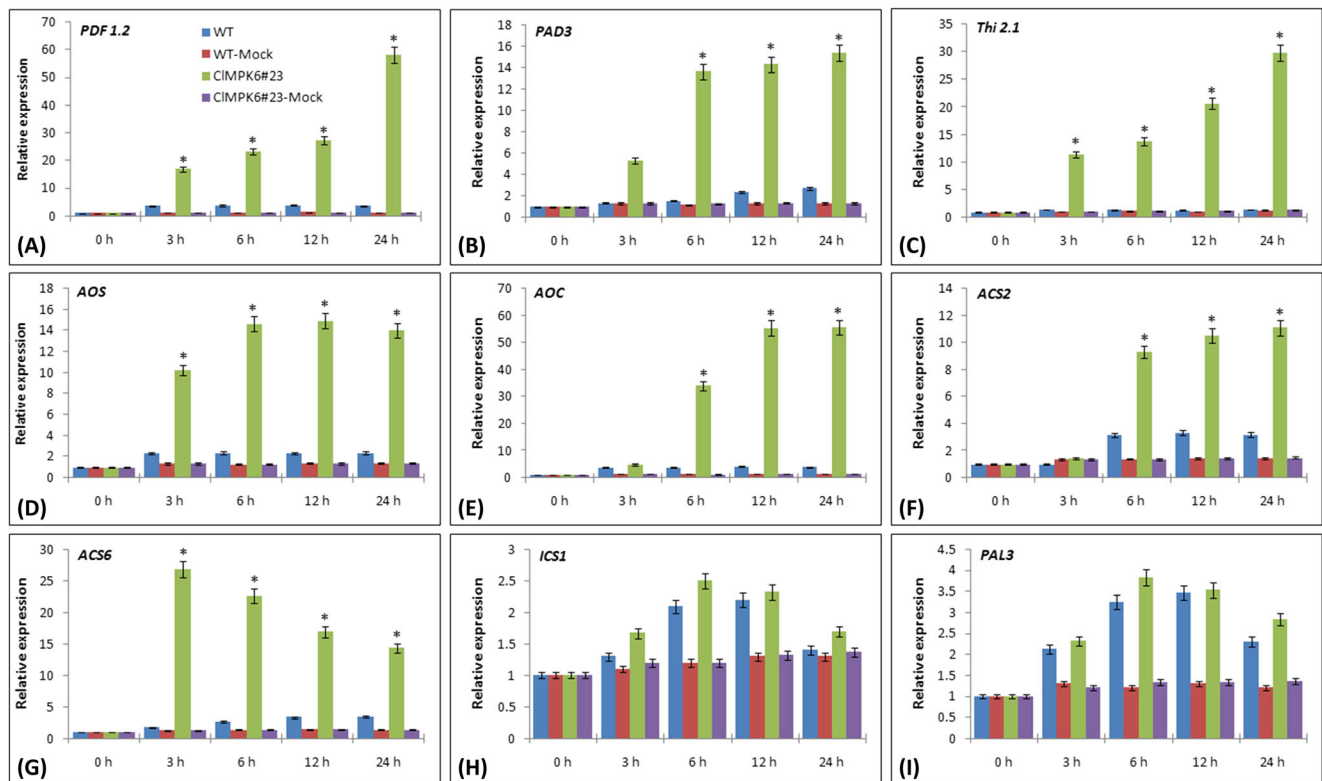


Fig. 8 Relative transcript levels of defense responsive genes in wild type (WT) and transgenic *Arabidopsis* line *CIMPK6#23*. Expression levels are shown as relative those of the control plants (0 h), which were set to 1. Standard error bars for the fold changes determined by qRT-PCR are shown. ANOVA test was used to determine the significance of difference between infected and mock samples. Asterisk indicates significant

difference at a *P* value < 0.05. The defense responsive genes analyzed are as follows: **a** *PDF 1.2*, *Plant Defensin 1.2*; **b** *PAD3*, *Phytoalexin deficient 3*; **c** *Thi 2.1*, *Thionin 2.1*; **d** *AOS*, *Allene oxide synthase*; **e** *AOC*, *Allene oxide cyclase*; **f** *ACS2*, *ACC synthase 2*; **g** *ACS6*, *ACC synthase 6*; **h** *ICS1*, *Isochorismate synthase 1*; and **i** *PAL3*, *Phenylalanine ammonia lyase 3*

pathogen-induced ethylene biosynthesis (Han et al. 2010; Li et al. 2012). Genetic analysis in *Arabidopsis* has shown that *ACS2* and *ACS6* are substrate of AtMPK6 whose phosphorylation leads to increase cellular ACS activity and ethylene production (Li et al. 2012). Therefore, it can be suggested that the regulation of ACS activity by *CIMPK6* at the transcriptional level might play a key role in determining the magnitude of ethylene induction after *Botrytis* infection.

SA-regulated signaling molecules often cross talk with JA components causing antagonistic response to necrotrophic infection (Gimenez-Ibanez and Solano 2013). Hence, we also examined the transcript levels of isochorismate synthase 1 (*ICS1*) and phenylalanine ammonia lyase 3 (*PAL3*), two important SA biosynthetic genes in the WT and transgenic plant after *Botrytis* infection. All the three transgenic plants and the WT used in the present study showed a small increase in the transcript levels of *ICS1* (1–2.5-folds) in both the infected and uninfected condition (Fig. 7h; S3 and S4). The expression of *PAL3* was almost similar and the transcript accumulation was insignificant in both WT and transgenic plants throughout the time course of infection (Fig. 8i; S3 and S4). This suggests a possible SA-independent regulation of defense response against *Botrytis* infection in *CIMPK6* transgenic plants. This makes sense as SA signaling is generally involved in the activation of defense responses against biotrophic and hemibiotrophic pathogens (Bari and Jones 2009). However, recent evidences of synergistic interactions between SA and JA defense pathway also suggest their possible association with defense against necrotrophic pathogens (Mur et al. 2006). Collectively, these results suggest that *CIMPK6* intervenes in several signaling pathways and is responsible for the induction of various defense responsive genes. Further in depth study is needed through generation of *CIMPK6* knockout mutants to explain its mode of action toward broad spectrum defense response against necrotrophic infection in plants.

In conclusion, a novel MAP kinase gene, *CIMPK6* responsive to *P. aphanidermatum*, was identified and characterized from turmeric. The expression of *CIMPK6* was significantly induced by MeJA, suggesting its involvement in a JA-dependent defense signaling. The present study also demonstrated the pathogen-induced priming of defense responses in *Arabidopsis* plants having ectopic expression of *CIMPK6*. We are currently investigating the upstream kinases such as *MKKs* and *MEKKs* associated with *CIMPK6* subset as well as the regulatory molecules linking *CIMPK6* and gene expression. Our results validate the characteristics of *AtMPK6* orthologs in defense against necrotrophic infection in plants.

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