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Molecular cloning and expression analysis of four turmeric MAP kinase genes in response to abiotic stresses and phytohormones

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Abstract

Plant mitogen activated protein kinase (MAPK) cascades comprise a complex network playing a major role in regulating extracellular stimuli as well as developmental processes. The present study involves cloning four *MAPKs* (*ClMPK1, 3, 4* and *5*) from *Curcuma longa*. All four *ClMPKs* have fully canonical motifs of MAPK and each is represented by a single copy in the turmeric genome. The analysis of exon-intron junctions revealed conserved nature of *ClMPKs* across different plant groups. The RT-qPCR analysis showed their expression in mature plant tissues. The transcript analysis using the RT-qPCR shows that the four *ClMPKs* were differentially regulated by cold, salinity, and drought stresses. *ClMPK4* showed a significant upregulation in the presence of NaCl, polyethylene glycol, and mannitol. The time-course expression analysis revealed a marked accumulation of *ClMPK1* and *ClMPK4* transcripts after mechanical wounding or applications of abscisic acid, H_2O_2, methyl jasmonate, and salicylic acid. *ClMPK5* showed a unique and pronounced expression in response to hexavalent chromium (Cr^{VI}).

Additional key words: abscisic acid, ClMPKs, chromium, cold, *Curcuma longa*, methyl jasmonate, RT-qPCR, salinity, water stress.

Introduction

The exposure of plants to a wide variety of biotic and abiotic stresses has made them to develop mechanisms to recognize external signals and undergo adaptive responses. The protein kinase catalyzed phosphorylation and dephosphorylation is a key method in controlling intracellular responses to extracellular signals (Jonak *et al.* 2002). The mitogen-activated protein kinase (MAPK) cascades are the chief categories of protein kinases in eukaryotes that play a dominant role in plant signal transduction (Taj *et al.* 2010, Sinha *et al.* 2011). This signaling cascade consists of three subsequently acting protein kinases: MAPK kinase kinase (MAPKKKs/ MEKK), MAPK kinase (MAPKKs/MKKs), and MAPK. MEKKs activates MKKs through phosphorylation of two serine/threonine residues in a conserved S/T-X3-5-S/T motif of the MKK activation loop which in turn activates MAPKs through dual phosphorylation of Thr and Tyr residues within a T-X-Y motif in the activation loop between kinase domains VII and VIII. The activated MAPKs in turn cause phosphorylation of transcription factors and other nuclear and cytosolic signaling units for controlling the expression of downstream genes (Suarez-Rodriguez *et al.* 2010).

The MAPK cascade has received a great attention for its crucial role in regulating cell differentiation, hormone signaling, responses to biotic and abiotic stresses, *etc*. (Mishra *et al.* 2006, Taj *et al.* 2010). The *Arabidopsis* MKK1/MKK2-MPK4/MPK6 cascade has a role in salt and cold stress signaling and it is rapidly activated by wounding (Ichimura *et al.* 2000). The activation of *MEKK1-MKK4/MKK5* and *MPK3/MPK6* cascades also increases the expression of transcription factor conferring resistance to pathogens (Asai *et al.* 2002). Rice *OsMPK14* and *Arabidopsis AtMPK4* also negatively regulate pathogen challenge (Pitzschke *et al.* 2009, Lee *et al.* 2011). Rice MAPK *OsMSRMK2* exhibits

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Abbreviations: ABA - abscisic acid; ClMPK - *Curcuma longa* mitogen activated protein kinase; MAPK - mitogen-activated protein kinase ; MeJa - methyl jasmonate; PEG - polyethylene glycol; RT-qPCR - real time-quantitative polymerase chain reaction; SA - salicylic acid.

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expression under a high concentration of heavy metals (Agrawal *et al.* 2002). Besides, MAP kinases also regulate plant growth and development. Tobacco MAPK *ntF4-NtMEK2* plays an active role in pollen maturation (Voronin *et al.* 2004), whereas *Arabidopsis AtMPK3* regulates stomatal development and transpiration (Gudesblat *et al.* 2007). Tobacco MAPK (SIPK) responds to plant growth and fitness by modulating salicylic acid (SA) and menthyl jasmonate (MeJA) cross talk (Meldau *et al.* 2012), and alfalfa MKK3 presumably controls plant cytokinesis (Laszlo *et al.* 1999). Further, a recent investigation revealed that MAPK also mediates priming plant cells, *i.e.*, response to a lower concentration of stimulus in a more vigorous and swift manner. The accumulation of *AtMPK3* and *AtMPK6* also causes priming during development of chemically induced resistance in *Arabidopsis thaliana* (Becker *et al.* 2009).

Functional genomics has led to the characterization of more than 60 MAPKs from different model plant species, such as *Arabidopsis*, rice (Hamel *et al.* 2006), poplar (Nicole *et al.* 2006), maize (Wu *et al.* 2011), and more recently *Brachipodium distachyon* (Chen *et al.* 2012) and tobacco (Zhang *et al.* 2013). However, it is necessary to isolate and identify more MAPKs from different plant species to get insights on specific structural and regulatory patterns of these signaling kinases.

Turmeric (*Curcuma longa* Loeb.) of the family *Zingiberaceae* is one of the important crops in tropical and sub-tropical countries with great medicinal and economic significances (Ravindran *et al.* 2007). However, the obligatory asexual nature, high stigmatic incompatibility of the turmeric lines, and uninterrupted domestication of the chosen genotypes made them highly prone to major biotic and abiotic stresses. The characterization of MAPKs in turmeric can provide information on the possible mechanism of various stress responses in asexually reproducing plants. So far, no report is available on MAPK cascades and their role in mediation of signal responses in turmeric. Our earlier study resulted in the identification of four MAPK partial sequences through the *in silico* analysis of the *Curcuma longa* EST database (Joshi *et al.* 2011). In the present study, we report the first cloning, characterization, and genomic organization of four *MAPK* genes. Further, we studied their expression in response to various stresses and phytohormones using RT-qPCR.

Materials and methods

Plants and treatments: Rhizomes harvested from mature plants of *Curcuma longa* Loeb. cv. Surama were sprouted in earthen pots and allowed to grow in a growth chamber at a temperature of 28 °C, a relative humidity of 80 %, a 14-h photoperiod, and an irradiance of 200 μmol m^{-2} s^{-1} for two months. Then the plants were immersed in different solutions: 100 μM methyl jasmonate (MeJA), 100 μM abscisic acid (ABA), 25 mM hydrogen peroxide (H_2O_2), 500 μM salicylic acid (SA), and 100 μM sodium nitroprusside (SNP). A treatment with pure water served as control. A salt stress was imposed by placing the roots in a 200 mM NaCl solution. Simulated drought was realized by treating with 10 % (m/v) polyethylene glycol (PEG 1 000) or 200 % (m/v) mannitol. A cold treatment was performed by placing the seedlings to 4 °C. A heavy metal stress was simulated by a 10 mM Cr^{VI} solution. The leaf lamina was cut with a sharp blade for a wounding treatment. Leaf samples were harvested at 0, 15, and 30 min, and at 1, 3, 6, 12, and 24 h, frozen in liquid nitrogen and stored at -80 °C until use. Roots, rhizomes, and leaves of 15-d-old and 3-month-old plants were collected separately for RNA isolation and used for organ specific expression analysis.

DNA and RNA isolation: Leaf tissue was frozen in liquid nitrogen and grounded into a fine powder. Total genomic DNA was extracted using the protocol described by Doyle and Doyle (1990). RNA was isolated from leaves using *TRI* reagent (*Sigma-Aldrich*, St. Louis, USA) following the manufacturer's instructions. The quality and content of DNA and RNA in samples were examined on an ethidium bromide-stained agarose gel (1.2 %, m/v, denaturing formaldehyde gel in case of RNA) and by measuring absorbances at 260 and 280 nm using a UV-VIS spectrophotometer (*Thermo*, UK).

RT-PCR and cloning turmeric MAPKs: Four partial turmeric MAPK sequences with high homology to known plant MAPKs as obtained from the *in silico* analysis of turmeric EST data (Joshi *et al.* 2011) were selected for further study. Full lengths of the gene sequences were obtained by the 5' rapid amplification of cDNA ends (5'-RACE) according to Frohman *et al.* (1988) with slight modifications. cDNA was synthesized from 2 μg of total RNA using the *GoScript* reverse transcription system (*Promega*, Madison, USA). The first strand cDNA synthesis was carried out using 2 ug of DNA free RNA primed with 15-mer oligo dT and 200 units of *MMuLV* reverse transcriptase (*Promega*) following the manufacturer's instruction and used as template for subsequent PCR. The reaction conditions consisted of one cycle at 25 °C for 5 min, followed by 42 °C for 30 min, and 85 °C for 5 min. The first-strand cDNAs were diluted 10-fold with nuclease-free water and stored at -80 °C for further use. Based on the available partial cDNA sequences, primers were designed for 5' RACE reactions (Table 1 Suppl.). The first round of PCR was followed by a nested round of PCR. The PCR amplification was programmed on a *Veriti* thermal cycler (*Applied Biosystems*, Carlsbad, USA) with 35 cycles at 94 °C for 30 s, 68 °C for 30 s, 72 °C for 2 min, with a final extension at 72 °C for 10 min. The amplified

products were cloned using a pTZ57R/T vector (*Insta clone T/A* cloning kit, *Fermentas*, Baden-Wurttenberg, Germany) and sequenced using a *BigDye* terminator cycle sequencing kit (*Perkin Elmer,* Norwalk, USA) on an *ABI Prism 3730* genetic analyzer (*Applied Biosystems*). The RACE-PCR product sequence revealed an expected overlap with the original EST sequence. Full-length cDNA of turmeric MAPKs were obtained by a reverse transcriptase PCR amplification with cDNA specific primers nearest to the 5' or 3' end. The PCR amplification was performed in a final volume of a 25 mm^3 reaction mixture containing 2 mm^3 of 25 mM $MgCl_2$, 0.2 mm^3 of a 10 mM dNTP mix, 5 mm^3 of a 5× reaction buffer, 1 mm^3 of synthesized cDNA, 1 U of *Taq* DNA polymerase, and 1 µM each of the MAPK specific primers. The reaction conditions were: 94 °C for 5 min followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 57 °C for 30 s, and elongating at 72 °C for 2 min, followed by a final extension at 72 °C for 7 min. The RT-PCR reaction included turmeric *actin 1* gene as positive control and RNA instead of cDNA as template for negative control. Amplicons were separated on a 1.2 % (m/v) agarose gel.

DNA sequencing and sequence analysis: The amplified products of expected size were purified using a *Wizard SV* gel and the PCR cleanup system (*Promega*) and cloned into a pTZ57R/T vector (*Insta clone T/A* cloning kit, *Fermentas*) following manufacturer's instructions. The recombinant vectors were transformed into competent *Escherichia coli* JM109 strain cells. The *Wizardplus* miniprep DNA purification system (*Promega*) was used to purify plasmid DNA followed by sequencing using a *BigDye* terminator cycle sequencing kit (*Perkin Elmer*). Sequences of the PCR product were determined on an *ABI Prism 310* genetic analyzer (*Applied Biosystems*). Sequences were edited using the *GeneDoc* software to remove the primer and vector sequences. Similarity searches were performed using *BLASTp* from the National Center for Biotechnology Information database (http://www.ncbi.nlm.nih.gov). Sequence translations were performed using the nucleotide translate tool at *ExPASy* server (http://www.expasy.ch). Sequence alignments were done using the *CLUSTALX* multiple sequence alignment tool at the European Bioinformatics Institute (http://www.ebi.ac.uk) with default settings. A phylogenetic analysis was performed based on the neighbor-joining (NJ) method with 1 000 bootstrapping replicates using the molecular evolutionary genetics analysis (*MEGA 6, v. 5*) package (Tamura *et al.* 2013). Conserved motif structures of predicted MAPKs were analyzed using the multiple expectation maximization for motif elicitation (*MEME*) (Bailey *et al.* 2006).

Genomic structure analysis of turmeric MAPKs: Gene-specific primers designed based on the 5' and 3' untranslated regions of cDNA sequences were used for the PCR amplification of the genomic clone of the turmeric MAPKs. PCR was carried out using 50 ng of genomic DNA in a 25 mm^3 reaction volume containing 2.0 mM $MgCl_2$, a 200 µM dNTP mix (*Fermentas*), a 10× PCR buffer, and 1.5 units of *Taq* DNA polymerase (*Merck Biosciences*, Darmstadt, Germany). The PCR conditions were 94 °C for 2 min followed by 35 cycles at 94 °C for 45 s, 55 °C for 45 s, 72 °C for 2 min, and a final extension at 72 °C for 15 min. The exon/intron structures of the MAPKs were determined by comparing the genomic clones with the cDNA sequences.

For a Southern blot analysis, 10 µg of *C. longa* genomic DNA was digested with restriction endonucleases: *EcoRV, BamHI, XbaI, BglII*, and *SacI* (*Fermentas*). The digests were electrophoretically separated on a 1 % (m/v) agarose gel, capillary-blotted onto a nylon membrane filter *(Hybond^{N+}*, *Amersham Pharmacia Biotech*, Piscataway, USA) with a 0.5 M NaOH transfer buffer, and baked at 80 °C for 2 h. Digoxigenin labeled probes were prepared from purified DNA of all the four turmeric MAPKs using a digoxigenin DNA labeling and detection kit (*Roche Diagnostics*, Basel, Switzerland). Gene specific probes were designed based on the respective 3' non-coding regions of the turmeric *MAPKs*. An approximately 300 bp fragment taken from the 3' untranslated regions (UTR) of each turmeric *MAPK* was PCR amplified as DIG-labelled probe using a mixture of DIG-labelled and standard dNTPs in a 1:3 ratio. The membrane was blocked at 62 °C for 1 h with *DIG Easy Hyb* (*Roche*) before hybridization. It was then hybridized with DIG-labelled probes at 65 °C for 15 h in a hybridization chamber followed by two washes with 0.1× SSC (15 mM NaCl, 1.5 mM Na-citrate, pH 7.0) containing 0.1 % (m/v) sodium dodecyl sulphate at 65 °C for 20 min. Detection of the hybridized probe was done according to the manufacturer's instructions.

Expression analysis through real time quantitative PCR (RT-qPCR): A pair of gene specific primers was designed for each of the four *ClMPK* genes (Table 2 Suppl.). The specificity of the primers was confirmed by running the RT-qPCR amplified products in an agarose gel before they were used in a quantitative real time PCR analysis. The RT-qPCR was set in a total volume of 25 mm^3 containing 12 mm^3 of a *SYBR Green* PCR reagent mix (*Applied Biosystems*), 1 mm^3 of each of the forward and reverse gene specific primers (20 µM), 7 mm^3 of PCR water, and 4 mm^3 of each reverse transcribed cDNA product. The RT-qPCR was carried out in a *StepOne* real time PCR system (*Applied Biosystems*). The PCR program had an initial denaturation step at 95 °C for 10 min followed by 40 cycles consisting of denaturation at 95 °C for 15 s, annealing at 60 °C for 40 s, and extension at 72 °C for 25 s. A dissociation curve was created at the end of each reaction using a cycle consisting of denaturation at 95 °C for 20 s and annealing at 60 °C for 40 s followed by a slow temperature increase to 95 °C at the rate of

0.2 °C s^{-1}. This was done to detect the presence of a primer-dimer and other unwanted amplified products that would negatively bias the results. For quantification of the PCR products, the constitutively expressed house-keeping gene *actin 1* of turmeric was used as endogenous control. The relative gene expression was calculated using the threshold cycle ($2^{-\Delta\Delta C_T}$) method (Pfaffl 2001). Five replicates were taken for each biological sample and an average value with a standard deviation was reported.

Comparative expression analysis of *ClMPKs* with corresponding *AtMPKs*: The available expression data of the corresponding MAPKs from *Arabidopsis* were downloaded from the *Genevestigator* database (Zimmermann *et al.* 2004) for comparison with the expression profiles of *ClMPKs*. For abiotic stresses and hormonal treatments, expression patterns of all samples were transformed into a log 2-based ratio and normalized for standardization of array data. An expression of a gene was defined with a log 2-based ratio higher than 0.5 or lower than -0.5 and a significant difference in the gene expression was determined. For *ClMPKs*, the gene expression was determined based on RT-PCR results.

Table 1. Molecular properties of the *ClMPKs* isolated from *Curcuma longa*, and sequences with significant homology to turmeric MAPKs as obtained through a *BlastP* search.

	Length of genomic DNA and cDNA [bp]	Exons/ introns	Number of amino acids	pI/Mr [kDa]	*BLAST* aligned sequence	Max. identity [%]	E-value
ClMPK1	2169/1110	6/5	369	5.62/42.262	MAPK 1 (*O. sativa*) (BAD34534)	91	0.0
ClMPK5	1861/1110	6/5	369	5.89/43.105	MAPK 5 (*O. sativa*) (AAL87689)	98	0.0
ClMPK3	1186/1104	2/1	367	6.94/42.058	MAPK 3 (*O. sativa*) (AAG40581)	98	0.0
ClMPK4	1191/1101	2/1	366	6.31/42.070	MAPK 7 (*Z. mays*) (ABC02871)	98	0.0

Results

Primers designed based on the sequence information of the turmeric ESTs resulted in cloning stress responsive MAPKs from *Curcuma longa*. RT-PCR using the primers resulted in isolating four full-length genes namely *ClMPK1*, *ClMPK3, ClMPK4*, and *ClMPK5* (Genebank acc. Nos. KC907274, KC907276, KC907277, and KC907275, respectively) (Fig. 1 Suppl.). Table 1 represents the genes, their encoded polypeptide lengths, calculated molecular mass, and isoelectric points.

The multiple sequence alignment shows the turmeric MAPKs shared a significant degree of sequence identity with known MAPKs from other plant species (Fig. 2 Suppl.). The deduced amino acid sequences of the four *ClMPKs* held a fully canonical motif with the 11 conserved sub domains that are characteristics of MAPKs and carry a dual phosphorylation activation motif (TEY: Thr-Glu-Tyr) located between subdomains VII and VIII. *ClMPK1* and *ClMPK5* represented a short C-terminus containing common docking (CD) domain with the consensus sequence DxxDE(P)xC (x represents any amino acid). *ClMPK3* and *ClMPK4* also had a conserved CD domain albeit a different one. To analyze the structural diversity of the conserved motifs in the turmeric MAPKs, we predicted the conserved motifs and their relative positions. The *MEME* motif detection software identified 15 motifs from the ClMPK proteins, 11 of which corresponded with the I-XI domains found in serine/threonine protein kinases (Fig. 1*A*,*B*). The sub domain VIII with the TEY signature, and the sub domain VII of the serine/threonine protein kinases corresponded with motif 1 and motif 2, respectively, and exhibited the highest conservation among the turmeric MAPKs. Likewise, the sub domain I with the conserved signature PIGRGAYGIVCS and the sub domain XI with the MLTFDPRQRI conserved amino acid sequence corresponded with motif 4 and motif 10, respectively. The *MEME* analysis also revealed the presence of an extra motif 13 in ClMPK3 and ClMPK4 besides the common motifs shared by ClMPK1 and ClMPK5.

The phylogenetic tree constructed based on turmeric MAPKs and 23 MAPKs from other plant species classified them into group A and group C of the MAPK family (Fig. 2). Group A contains *ClMPK1* and *ClMPK5* with a 72 % homology between each other. *ClMPK1* exhibited a 91 % sequence identity to *OsMPK1* from *Oryza sativa*, 86 % to *ZmMAPK5* from *Zea mays*, 85 % to *TaMAPK1* from *Triticum aestivum*, and 82 % to *AtMPK6* of *Arabidopsis thaliana* of group A MAPKs. Similarly, *ClMPK5* also shared a high homology with group A MAPK members (a 98 % sequence identity to *OsMPK,* 83 % to *ZmMAPK4*, and 75 % to *AtMPK3*). On the other hand, group C includes *ClMPK3* and *ClMPK4* with a 96 % sequence identity between each other. *ClMPK3* revealed the highest similarity with *OsMPK3* (98 %) and *AtMPK1* (83 %), whereas *ClMPK4* was closest to *ZmMPK7* (98 %) and *AtMPK2* (83 %).

The copy number of *ClMPKs* in the turmeric genome was estimated using the Southern blot analysis. Since the mRNA encoded by the four turmeric MAPKs had a high degree of sequence conservation and size similarity,

small size probes were designed to avoid cross hybridization between family members. Under low stringency conditions, the DNA sample digested with *EcoRV, BamHI, XbaI, BglII*, and *SacI* restriction enzymes detected a single hybridization signal for all the four turmeric MAPKs (Fig. 3*A*). This indicates that the genes encoding ClMPKs were apparently present as single-copy genes in the turmeric genome.

The exon/intron organization for the *ClMPKs* revealed vital information on the gene structures and the evolutionary pattern of the MAPK gene family in turmeric. The alignment of the genomic fragment with the corresponding cDNAs established the genomic structures of the *ClMPKs*. *ClMPK1* and *ClMPK5* consisted of six exons and five introns of conserved or variable sizes. On the other hand, *ClMPK3* and *ClMPK4* of group C were composed of only two exons with strictly conserved or very similar sizes (Fig. 3*B*). The comparative analysis of exon-intron junctions between the *ClMPKs* and their corresponding orthologs from *Oryza sativa, Zea mays, Arabidopsis thaliana*, and *Populus trichocarpa* showed well conserved exons, whereas intron lengths varied among species (Fig. 3 Suppl.). All the introns were A+T rich with an elevated T content with the 5' and 3' splice junctions representing a canonical consensus di-nucleotide sequence GT-AG which is a typical structural characteristic of plant introns. An intron phase analysis indicates that the single intron of both *ClMPK3* and *ClMPK4* was within phase 0. Similarly, the three middle introns of both *ClMPK1* and *ClMPK5* were within phase 0, whereas the first and the fifth intron were within phase 2 and phase 1, respectively.

RT-PCR confirmed the transcription of *ClMPKs* in leaves, roots, and rhizomes of both young and mature plants. The expressions of the four *ClMPKs* were higher in leaves than in rhizomes and roots (Fig. 3*C*). *ClMPK1* and *ClMPK5* showed the least expression in rhizomes, whereas *ClMPK3* and *ClMPK4* in mature roots. *ClMPK1*

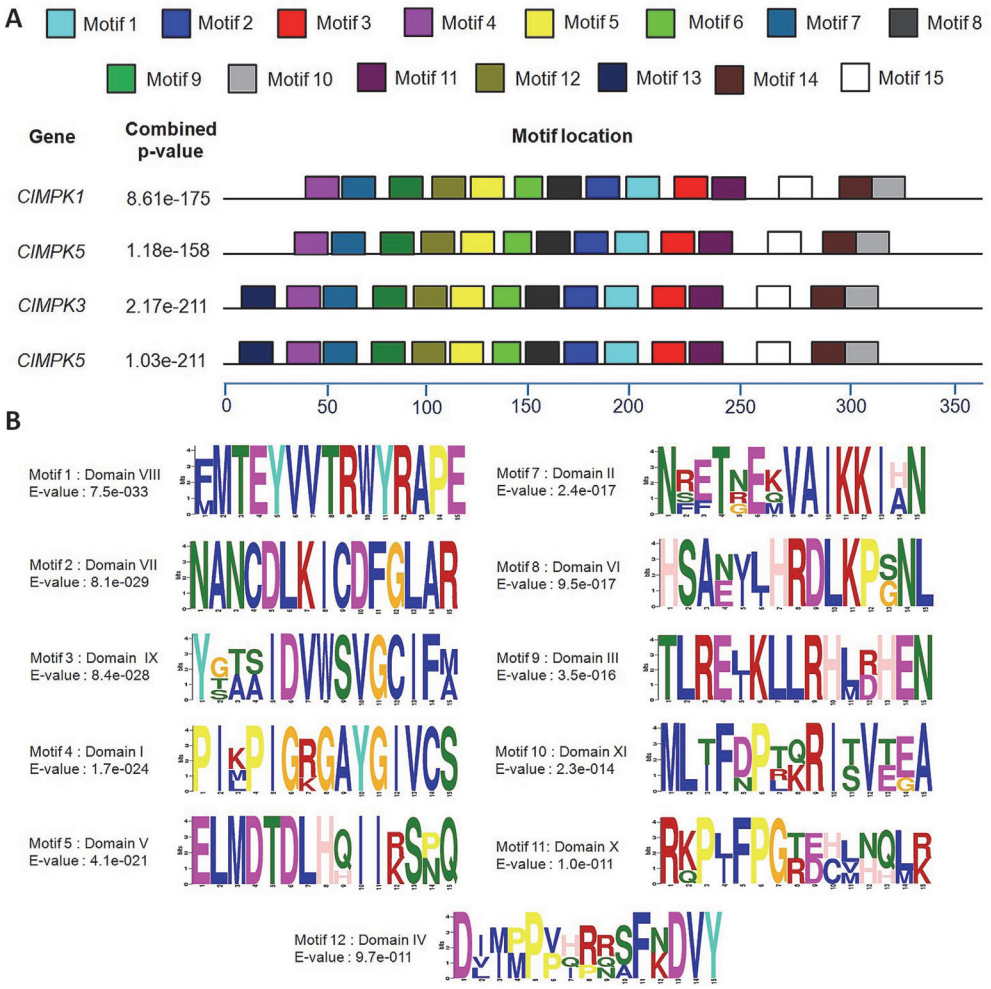

Fig. 1. *A* - The schematic representation of amino acid motifs of four turmeric MAPKs as analyzed through the *MEME 4.0* software tool. *Black solid line* represents different *ClMPKs* and their length whereas differently *colored boxes* represent different conserved motifs along the length of each *ClMPK*. *B* - The sequence logos of 11 conserved motifs corresponding to the 11 characteristic domains of the *MAPK* gene along with their E-values.

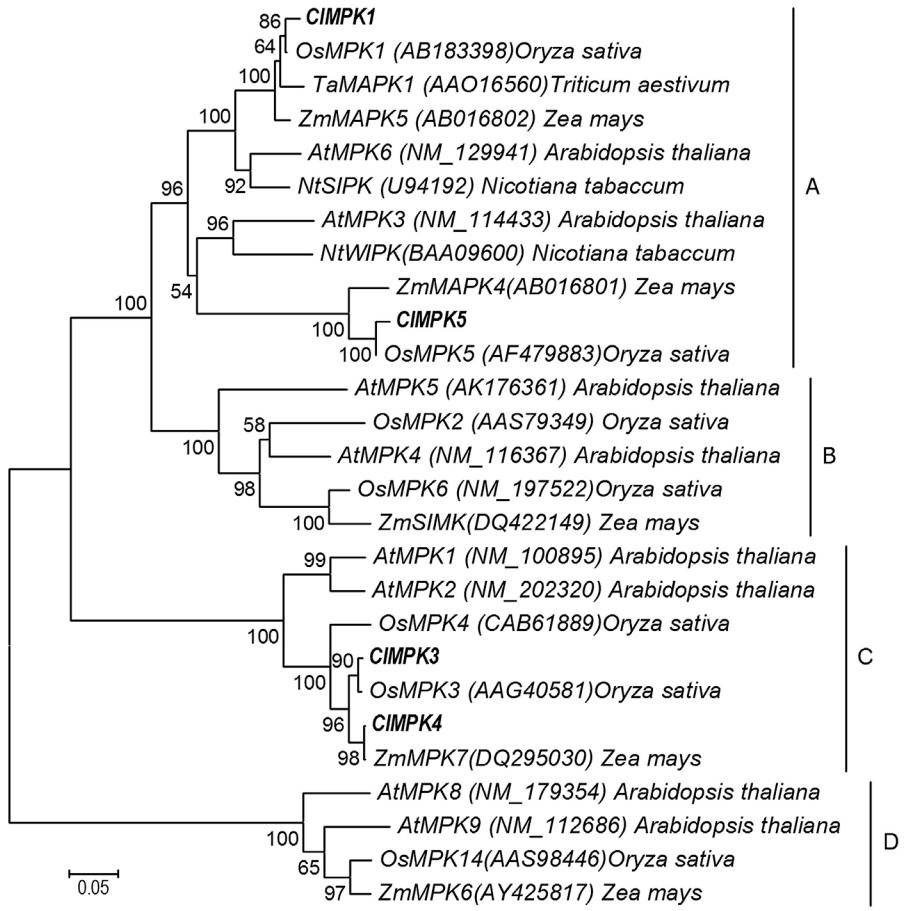

Fig. 2. The phylogenetic relationship of four *ClMPKs* with other MAPK family members from different plant species. The phylogenetic tree was constructed by the neighbor-joining method using the *MEGA 5.2* program. Numbers on the branches indicate the percentage of 1 000 bootstrap replications supporting the particular nodes. Letters A-D on the right indicate different groups of MAPKs.

Table 2. The transcription of four *ClMPKs* in response to cold (4 °C), salinity (200 mM NaCl), and a heavy metal stress (10 mM Cr^{VI}) analyzed by RT-qPCR. Data were normalized using a turmeric *actin* gene. Samples were taken at 0, 15, and 30 min, and at 1, 3, 6, 12, and 24 h after the treatments. Means ± SD, $n = 5$.

	0 min	15 min	30 min	1 h	3 h	6 h	12 h	24 h
Cold								
ClMPK1	0.640±0.071	1.071±0.043	5.118±0.066	1.121±0.061	1.070±0.063	1.146±0.039	1.056±0.101	1.057±0.052
ClMPK3	0.740±0.062	1.271±0.074	1.011±0.036	4.897±0.053	0.944±0.112	1.105±0.057	2.846±0.063	0.978±0.098
ClMPK4	0.840±0.067	1.012±0.035	6.170±0.087	0.897±0.074	1.094±0.046	1.105±0.104	1.846±0.036	1.098±0.025
ClMPK5	0.440±0.069	1.011±0.031	0.832±0.079	0.807±0.064	0.644±0.041	0.612±0.093	0.713±0.114	0.486±0.083
Salinity								
ClMPK1	0.532±0.059	1.064±0.039	0.966±0.082	0.923±0.042	0.882±0.031	0.617±0.060	0.532±0.072	0.855±0.047
ClMPK3	0.512±0.121	0.828±0.072	1.382±0.168	0.764±0.096	0.799±0.052	0.938±0.135	0.752±0.089	0.566±0.058
ClMPK4	0.514±0.103	1.043±0.053	1.382±0.132	4.764±0.081	1.389±0.032	0.922±0.102	0.852±0.091	0.866±0.063
ClMPK5	0.516±0.054	0.842±0.061	0.816±0.075	0.944±0.096	0.789±0.023	0.737±0.067	0.852±0.043	0.526±0.029
Heavy metal								
ClMPK1	0.558±0.045	0.652±0.024	2.842±0.061	1.048±0.109	0.863±0.071	0.546±0.133	0.506±0.059	0.446±0.032
ClMPK3	0.398±0.064	0.692±0.053	0.824±0.069	1.209±0.088	0.673±0.075	0.670±0.030	0.446±0.077	0.449±0.059
ClMPK4	0.598±0.121	0.689±0.072	0.821±0.168	2.775±0.096	1.235±0.052	0.771±0.135	0.646±0.089	0.696±0.051
ClMPK5	0.586±0.089	0.792±0.076	0.811±0.132	0.775±0.083	3.894±0.089	0.675±0.112	0.449±0.056	0.546±0.041

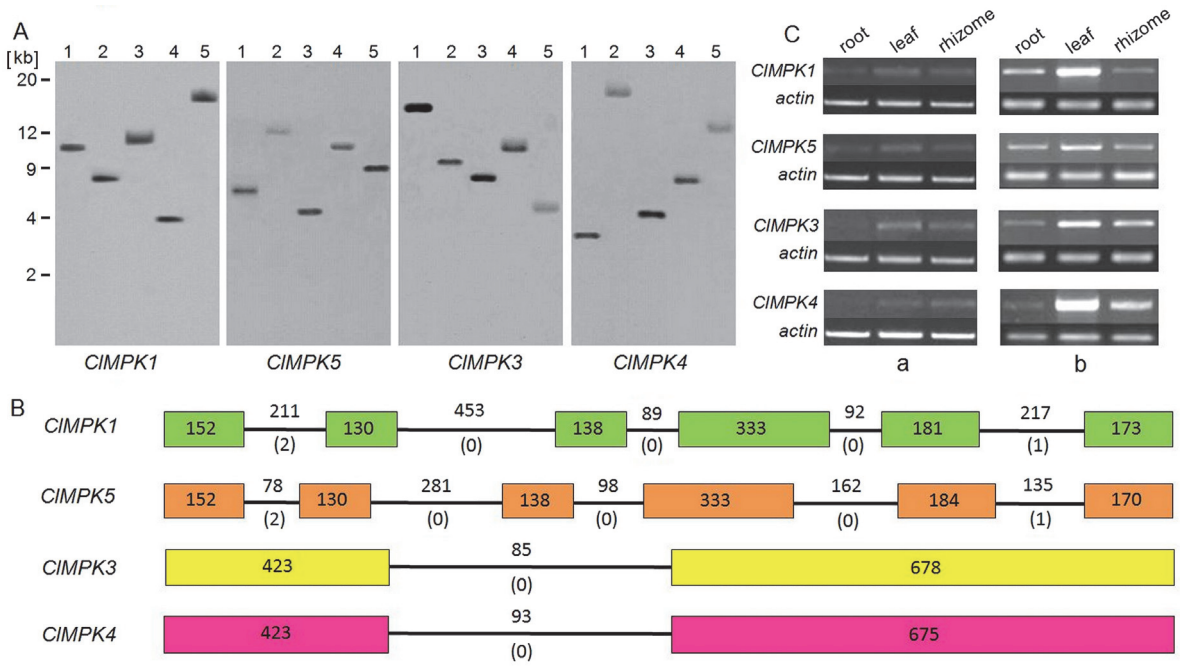

Fig. 3. *A* - The genomic organization of *ClMPKs*: 1 - *EcoRV,* 2 - *BamHI,* 3 - *XbaI,* 4 - *BglII*, and 5 - *SacI*. Genomic DNA of *Curcuma longa* was digested with the designated restriction enzymes, fractionated on a 0.8 % agarose gel, blotted onto a nylon membrane and hybridized with gene specific probes based on the respective 3' non-coding regions of the turmeric MAPKs. *B* - The intron-exon organization of *ClMPKs*. Introns are represented by *dark lines* and exons by *colored boxes*. The individual intron and exon lengths are given in base pairs. The numbers between brackets corresponds to the intron phase. *C* - The expression patterns of *ClMPKs* obtained through qRT-PCR in root, leaf, and rhizome tissues of *Curcuma longa* (a, b - the transcript expression in different tissues at the seedling and mature stages, respectively). *Actin* was used as reference gene for the expression analysis of *ClMPKs*.

and *ClMPK4* transcripts were more abundant than those of the other *ClMPKs* in all the three tissue types in the mature plants. On the other hand, *ClMPKs* expressed poorly in 15-d-old plants. *ClMPK1* and *ClMPK5* had no expression, whereas *ClMPK3* and *ClMPK4* had a negligible expression in roots of the young plants.

To determine the expression pattern of turmeric MAPKs in response to abiotic stresses, a RT-qPCR was carried out using RNA samples harvested at various intervals after subjections to low temperature (4 °C), NaCl, and Cr^{VI}. The most pronounced and fast effect of the cold stress was observed in the *ClMPK4* transcription which increased more than 6-fold within 30 min and then suddenly decreased to 1.1 after 1 h, rebound to a 2-fold increase after 12 h and finally coming down to the basal level within 24 h (Table 2). A fast and transient transcript accumulation was also observed in *ClMPK1*. The *ClMPK3* transcription was induced more than 5-fold only after 1 h under the cold stress, then gradually decreased until 6 h, followed by an increase again (2.5-fold) at 12 h, and finally reaching the basal level after 24 h. There was no significant upregulation of turmeric MAPKs in response to the salt stress except *ClMPK4* which recorded more than a 4.5-fold upregulation at 1 h. Likewise, only *ClMPK5* showed an upregulation more than 3-fold due to the heavy metal stress induced by Cr^{VI}. The treatment with Cr^{VI} also resulted in a 2.5-fold increase in the transcriptions of *ClMPK1* and *ClMPK4*.

PEG and mannitol can modify the osmotic potential of nutrient solution and thus induce plant water deficit. The transcriptions of *ClMPK1, ClMPK5*, and *ClMPK4* increased more than 3-fold due to the PEG or mannitol treatments (Fig. 4). The *ClMPK4* transcription increased almost 7-fold of the control within 1 h of the PEG treatment before decreasing to the basal level after 3 h. The *ClMPK5* transcription also increased within 1 h of the PEG treatment. *ClMPK1* revealed a 4-fold increase in the transcription albeit only at 3 h after the treatment. Due to the mannitol treatment, the *ClMPK4* transcription increased more than 7-fold within 30 min and then gradually decreased to 3-fold after 1 h, 2-fold after 6 h, and reached the basal level after 12 h. The *ClMPK1* and *ClMPK5* transcriptions increased 5-fold within 30 min and 1 h, respectively, before declining to the basal level.

The RT-qPCR analysis confirmed the role of the four turmeric MAPKs in mediating hormonal signaling. Physiological differences or circadian rhythms could be responsible for differential transcript accumulation at 0 h. After the application of H_2O_2, a potent reactive oxygen species, the transcriptions of *ClMPK3, ClMPK4*, and *ClMPK5* were significantly upregulated. *ClMPK5* revealed the fastest transcript accumulation (5-fold within 30 min) before downregulation of trancription to the basal level within 1 h (Table 3). *ClMPK3* also showed a fast and significant increase in the transcription, whereas *ClMPK4* had 5-fold increase in the transcription only after 3 h. Likewise, the treatment with MeJa, a dominant signal molecule of the wound signaling pathway, resulted

in the activation of the transcriptions of *ClMPK1, ClMPK3*, and *ClMPK5*. The *ClMPK1* transcription increased within 1 h of the MeJa treatment, whereas an 8-fold increase in the *ClMPK4* transcription was observed only after 3 h, and an increase in the *ClMPK3* transcription after 12 h. Abscisic acid (ABA) induced a significant accumulation of *ClMPK4* transcripts within 30 min of the treatment followed by a gradual decline up to 24 h. *ClMPK1* showed more than a 7-fold increase in the transcription after 3 h before declining to the basal level after 24 h. The *ClMPK5* and *ClMPK3* transcriptions increased 2-fold after 1 and 3 h exposures to ABA. This slight increase in the *ClMPK5* and *ClMPK3* transcriptions needs a further evaluation as two-fold increase is considered insignificant. SA, a potent signaling molecule involved in response to pathogenesis, also induced the *ClMPK1* and *ClMPK4* transcriptions. The *ClMPK1* transcription reached a peak within 30 min and declined to the basal level after 1 h (Table 3). *ClMPK4* showed the highest transcription after 1 h,

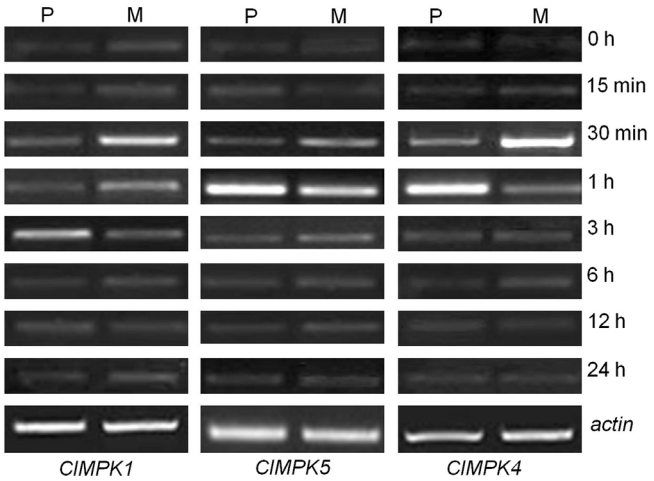

Fig.4. The expression analysis of *ClMPKs* in turmeric leaves treated with polyethylene glycol (P) and mannitol (M) using RT-qPCR. All RT-PCR reactions were from the same batch of cDNA and were repeated five times with identical results. Only one image of the replicates is shown here.

Table 3. The transcription of four *ClMPKs* induced by SA, ABA, MeJa, and H_2O_2. Samples were taken at 0, 15, and 30 min, and at 1, 3, 6, 12, and 24 h after the treatments. RT-qPCR data were normalized using the turmeric *actin* gene. Means ± SD, $n = 5$.

	0 min	15 min	30 min	1 h	3 h	6 h	12 h	24 h
Salicylic acid								
ClMPK1	0.740±0.071	1.102±0.041	8.928±0.043	1.002±0.068	1.048±0.082	1.446±0.077	2.058±0.089	1.411±0.081
ClMPK3	0.909±0.076	1.078±0.129	0.933±0.081	1.037±0.132	1.125±0.120	1.146±0.043	1.529±0.130	1.051±0.072
ClMPK4	0.709±0.076	1.078±0.113	0.933±0.086	8.977±0.029	1.125±0.114	2.347±0.041	1.488±0.136	1.451±0.078
ClMPK5	0.709±0.076	1.078±0.121	0.933±0.061	1.037±0.112	1.965±0.085	1.347±0.046	1.909±0.067	1.451±0.075
Abscisic acid								
ClMPK1	0.703±0.062	0.932±0.098	0.774±0.087	1.856±0.056	7.611±0.033	1.375±0.040	2.116±0.076	1.206±0.088
ClMPK3	0.807±0.056	1.028±0.066	1.137±0.045	1.294±0.042	1.862±0.056	1.264±0.049	1.236±0.077	1.120±0.071
ClMPK4	0.607±0.056	0.828±0.061	7.937±0.054	1.214±0.052	1.662±0.053	1.264±0.050	1.036±0.073	1.060±0.038
ClMPK5	0.607±0.059	0.828±0.061	1.097±0.059	1.974±0.019	1.322±0.032	1.264±0.056	1.436±0.051	1.100±0.061
Methyl jasmonate								
ClMPK1	1.552±0.075	1.042±0.027	0.956±0.096	13.028±0.061	1.119±0.137	1.691±0.067	2.403±0.103	1.784±0.073
ClMPK3	0.636±0.044	1.196±0.040	1.149±0.057	1.190±0.045	1.021±0.090	1.171±0.111	5.283±0.145	1.372±0.064
ClMPK4	1.036±0.036	1.496±0.051	1.229±0.137	1.790±0.043	8.221±0.086	1.671±0.106	1.283±0.131	1.372±0.076
ClMPK5	0.536±0.049	0.896±0.043	1.009±0.094	1.190±0.051	1.021±0.096	1.671±0.115	0.830±0.156	0.872±0.058
Hydrogen peroxide								
ClMPK1	0.842±0.052	1.326±0.041	1.511±0.030	0.652±0.075	0.278±0.030	1.463±0.041	1.655±0.314	2.031±0.147
ClMPK3	0.713±0.071	1.056±0.062	3.590±0.086	1.094±0.050	0.951±0.037	1.288±0.064	1.146±0.062	1.055±0.102
ClMPK4	0.803±0.062	1.356±0.086	1.590±0.050	0.994±0.037	5.451±0.064	1.288±0.062	1.846±0.106	1.355±0.121
ClMPK5	0.803±0.067	1.056±0.089	5.590±0.073	0.894±0.042	0.851±0.071	1.588±0.058	1.846±0.166	0.955±0.132

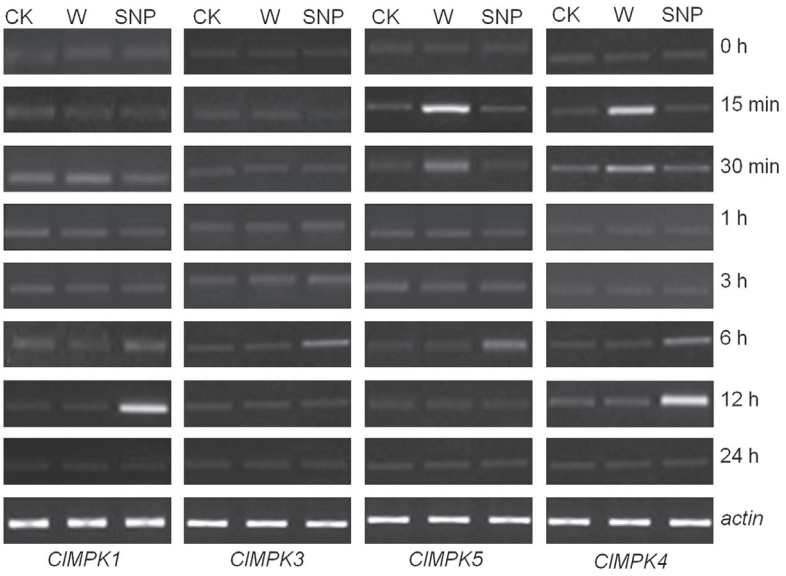

Fig. 5. The relative transcript expression of four *ClMPKs* in response to wounding (W) and sodium nitroprusside (SNP). All RT-qPCR reactions were from the same batch of cDNA and were repeated five times of which only one image is shown here.

declined to the basal level after 3 h, followed by a 2-fold increase after 6 h and by a gradual decline to the basal level after 24 h. The SA treatment had no significant impact on the expression patterns of *ClMPK3* and *ClMPK5*.

Upon wounding, the turmeric plant accumulated the *ClMPK4* and *ClMPK5* transcripts within 15 min which gradually decreased to the basal level after 6 h (Fig. 5). *ClMPK1* and *ClMPK3* revealed only minimal changes. The turmeric plants were also treated with SNP, and the *ClMPK1* and *ClMPK4* transcriptions increased after 12 h before declining to the basal state after 24 h. The SNP treatment resulted in only a 2-fold increase in the transcriptions of *ClMPK3* and *ClMPK5*.

Marked differences in the expression patterns of *ClMPKs* and their corresponding *AtMPKs* were observed under the different stresses. Although *AtMPK3* and *AtMPK6* underwent significant upregulations under the cold and salt stresses, no such activity can be seen in their corresponding turmeric MPKs (Table 3 Suppl.). On the other hand, three *ClMPKs* (*ClMPK1, ClMPK4*, and *ClMPK5*) showed pronounced expressions under the dehydration stress. The *AtMPK1* and *AtMPK2* expressions decreased under hormonal treatments, whereas their turmeric counterparts (*ClMPK3* and *ClMPK4*) were largely upregulated under similar treatments. However, *ClMPK5* and *AtMPK3* showed uniform expression profiles under the wound stress.

Discussion

In the present study, we cloned MAPKs from *Curcuma longa* using RT-PCR and designated them as *ClMPK1, ClMPK3, ClMPK4,* and *ClMPK5* based on their homology with known MAPKs from *Oryza sativa* and *Zea mays*. *ClMPK1* and *ClMPK5*, belonging to group A, possesses a CD domain in their C-terminal region. The CD domain is an integral characteristics of group A and group B MAPKs and often functions as binding site of MAPKKs. The analysis of genomic sequences of *ClMPKs* revealed highly conserved exons and introns. This suggests a strong negative selection for a change of MAPK protein sequences across different species. Further, the small variation in the exon length at the end of the coding sequences suggests rigid functional conservation of the centrally located catalytic domain of the protein kinase (Nicole *et al.* 2006).

The analysis of the tissue specific expression of *ClMPKs* is decisive for determining their roles in plant growth and development. There are several reports involving MAPKs in plant developmental processes (Huang *et al.* 2002, Wang *et al.* 2010). The four *ClMPKs* showed a high transcription in leaves of the mature plants but only weak in leaves of the young plants. This suggests that the *ClMPKs* might carry out unique signaling roles or developmental modules in leaf differentiation programming at late developmental stage. A pronounced accumulation of the *ClMPK1* and *ClMPK4* transcripts in the leaf tissue suggests that they were involved in the same signaling pathways, though they belong to different groups according to the amino acid sequences. A further detailed analysis of transcript accumulation at different stages of leaf development can throw more insight into their roles in leaf differentiation and physiology.

The RT-qPCR evaluated the expression pattern of turmeric MAPKs in response to the salinity, cold,

drought, and heavy metal stresses. Time course gene expression profiling showed a significant increase in the expressions of *ClMPK1, ClMPK3*, and *ClMPK4* within 30 min of the cold treatment. Cold stress upregulates many of group A and group C MAPKs from different plant species (Berberich *et al.* 1999, Ichimura *et al.* 2000, Wang *et al.* 2010, Chen *et al.* 2012). The qRT-PCR analysis also revealed a significant upregulation of *ClMPK4* within 1 h of the NaCl treatment before declining to the basal level. This is interesting because an earlier report show increased sensitivity to salt stress by an overexpression of group C MAPK OsMPK14 in rice (Lee *et al.* 2011). Thus, we can assume that *ClMPK4* might be involved in the cross talk with a MEKK1-MEK1 signaling cascade similarly to *Arabidopsis AtMPK7* (Menges *et al.* 2008). A detail cross talk analysis between upstream activator kinase and *ClMPK4* may throw new insights on the salt induced activation of *ClMPK4 in vivo* as well as *in vitro*. The treatment of the turmeric plants with Cr^{VI} resulted in the significant expression of *ClMPK5* within 3 h which is in agreement with a remarkable increase in the expression of group A MAPK *ZmMPK5* in *Zea mays* (Ding *et al.* 2009). An excess of heavy metals usually disrupts the cellular redox status leading to build-up of reactive oxygen species which can act as signal molecules for activation of MAPKs (Opdenakker *et al.* 2012). The transcripts of *ClMPK5* were also accumulated within 30 min of exposure to H_2O_2. Thus, the *ClMPK5* activation in response to Cr^{VI} might be largely mediated by ROS production. *ClMPK1, ClMPK5*, and *ClMPK4* displayed a pronounced expressions in response to the dehydration stress induced by PEG or mannitol. Mannitol or PEG induced many drought responsive MAP kinases in different plant species (Agrawal *et al.* 2002, Lee *et al.* 2011, Wang *et al.* 2010).

Increasing evidences suggest MAPK activation by different signal molecules, such as ABA, MeJA, SA, and H_2O_2 (Blanco *et al.* 2006, Zong *et al.* 2009, Shi *et al.* 2010). The positive regulation of *ClMPK1* and *ClMPK4* by the four signal molecules studied suggests the general role of phytohormones in plant signal transduction pathways leading to the activation of abiotic stress responses. Protein kinases control the signaling pathways towards the expression of defense related genes in different plants (He *et al*. 1999). *ClMPK1* and *ClMPK4* also exhibited a pronounced transcript upregulation in response to SA and MeJA, the two hormones that regulate major anti-herbivore and anti-pathogen defense responses, respectively. Kumar *et al.* (2009) reported the activation of *AhMPK3* in *Arachis hypogea* in response to MeJA treatment and wounding. The transcriptions of *ClMPK1* and *ClMPK4* were also increased after wounding. Wound responsive *p44MAPK* from forage grasses shows an enhanced transcription after adding SA and MeJa (Dombrowski *et al.* 2011). We are currently looking into the detailed expression analysis of the *ClMPKs* in the presence of *Pythium aphanidermatum*, an *oomycete* infecting the turmeric plant.

A comparative analysis of *ClMPKs* and *AtMPKs* suggests that during evolution the orthologous MPKs may show divergent function or may remain the part of the same ancestral function under different stress conditions. A previous study have also shown that expression profiles of some orthologous pairs of *BdMPKs* (*Brachypodium distachyon* MPKs) and *OsMPKs* (*Oryza sativa* MPKs) have uniform expression profiles, whereas few other vary (Chen *et al.* 2012). This functional differentiation can be attributed to gene duplication. Gene duplication may lead to pseudogenization causing loss of function or it may undergo neofunctionalization causing an upregulation or retention of the original function.

In conclusion, this is the first report of cloning and isolation of four distinct MAPKs from turmeric, an economically significant vegetatively propagated crop plant often affected by variable natural stresses. The structural characterization of *ClMPKs* identified them as single copy genes having similar pattern of exon/intron arrangements resembling their orthologs in the groups A and C of the MAPK family. The organ specific expression suggested their involvement in turmeric leaf growth and development. The expression profiles demonstrated the differential transcript regulation of turmeric MAPKs by different environmental factors and signaling substances. MAPK responses to multiple stresses largely depend upon the cross talk at the level of upstream parts of the MAPK cascades like MKKs and MEKKs. Until now, there is no report on the isolation of MKKs and MEKKs from turmeric or any other related species from *Zingiberaceae*. The detail elucidation and characterization of MAPK cascades and their components in turmeric will play a major role in development of new cultivars with biotic and abiotic stress tolerances.

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