Indian J. Hort. 69(2), June 2012: 145-150

Molecular cloning and characterization of a partial mitogen-activated protein kinase cDNA, *CnMAPK1* induced in response to water stress in coconut

Bobby Paul*, Rajesh M.K. and Naresh Kumar S.*
Central Plantation Crops Research Institute, Kasaragod 671 124, Kerala

ABSTRACT

Plants have developed a fine-tuned complex of stress responses, among which one of the earliest signaling pathways after perception of stimuli is the evolutionary conserved mitogen-activated protein kinase (MAPK) cascade. These cascades are major pathways by which extracellular signals are transduced into internal responses. In this study, oligonucleotide primers were designed based on conserved regions of plant MAPK sequences available in pubic databases. A cDNA clone of 467 bp encoding a MAPK (designated *CnMAPK1*) was isolated by RT-PCR from leaves of coconut plantlets subjected to water stress using polyethylene glycol (PEG) treatment. *CnMAPK1* was significantly homologous to other plant MAPKs *viz*., MPK7 and MPK5 from *Zea mays*, SIPK and MAPK6 from *Oryza sativa* and FLRS from *Triticum aestivum* (93% identity). Coconut MAPK belongs to the Serine/Threonine Kinases (STKs), Plant TEY MAPK subfamily group A. Molecular modeling of the coconut MAPK was also undertaken.

Key words: MAP kinase, water stress, coconut, cloning, molecular modeling.

INTRODUCTION

Availability of water and extreme temperatures are major limiting factors for optimal growth and development in plants. Plants respond and adapt to water stress through various biochemical and physiological processes. Numbers of genes that respond to abiotic stress at the transcriptional level have recently been described (Amudha and Balasubramani, 1). Mitogen-activated protein kinase (MAPK) cascades are universal signal transduction modules in eukaryotes. These protein phosphorylation cascades link extra-cellular stimuli to a wide range of cellular responses. In plants, MAPK cascades are associated with various physiological, developmental and hormonal responses. Molecular and biochemical studies have revealed the correlation of MAPK activation with pathogen infection, wounding, low temperature, drought, high salinity, touch and reactive oxygen species (Zhang and Klessig, 15; Morris, 10).

The coconut palm (*Cocos nucifera* L.) is one of the most extensively grown and used perennial oil seed and it plays a significant role in economic income and vegetable oil market (Harries, 3). Water deficit is the main environmental factor limiting coconut palm productivity (Murray, 11). Drought causes visible injuries to leaves and reduces the yield for several months (Rao, 12). In addition, water-stressed palms could be more sensitive to other biotic and abiotic stresses, which further limit productivity. Coconut generally withstands moderate drought, yet severe drought causes the frequent mortality in existing plantations (Karunaratne *et al*., 6). Being a perennial crop producing for more than 40 years and attaining bearing age in 3-7 years depending on the variety, the loss of palms has severe economic consequences. Therefore, it is important to identify relevant gene(s) and characterize the regulation in response to water stress in coconut. In this study, we isolated a cDNA sequence encoding a MAP kinase induced during water stress in coconut. Molecular modeling of the isolated MAP kinase was also undertaken.

MATERIALS AND METHODS

The nucleotide sequences of MAP kinase from different plant species were retrieved from GenBank (NCBI) and their sequence level similarity searches were performed using ClustalW. The phylogenetic analysis of MAP kinase was done and the similarity score above 50% were chosen for further primer designing and validation. The conserved regions from these sequences were selected for primer designing. The primers were checked for their quality (GC%, annealing temperature, presence of dimers) using the software Fast PCR.

Culture of mature embryos of coconut West Coast Tall cultivar was initiated. Embryos, along with a portion of the endosperm, were scooped out by means of a cork borer from the dehusked and split opened mature nut. Surface-sterilization of the excised embryos was done with 50% chlorine water for 20 min. and washing 4-5 times in sterile distilled

*Corresponding author's present address: Division of Environmental Sciences, Indian Agricultural Research Institute, New Delhi 110012; E-mail: nareshkumar.soora@gmail.com

water till the traces of chlorine were removed. The basal medium used for retrieval of the embryos was Y3 medium (Eeuwens, 2) supplemented with 30 g/l of sucrose. The embryos were sub-cultured to the same basal medium at monthly intervals. On germination of the embryos, 0.5 mg/l BAP and 0.5 mg/l NAA were supplemented in the medium for proper root and shoot development. When the plantlet reached the 3-4 leaflet stage, they were sub-cultured onto Y3 liquid medium containing 4 mg/l NAA and 2 mg/l BAP. The plantlets were sub-cultured onto the same medium once every 40 days.

Fully-grown *in vitro* plantlets (with 4-5 leaflets and fully developed root system) were sub-cultured onto basal medium containing 20% polyethylene glycol (PEG, 6000 MW). Leaf samples were collected after 10 days of inoculation of the plantlets in the PEG medium for isolation of RNA. Leaf samples were also collected from control plantlets grown in basal medium.

Total RNA was extracted using RNeasy plant mini kit (Qiagen, Germany) from both plantlets exposed to water stress and also unstressed, control plantlets. Total RNA was treated with *DNase* I to remove any traces of genomic DNA. The reaction mix contained 20 µg total RNA, 40 mM Tris-HCl (pH-7.5), 6 mM $MgCl_2$ and 5 U of *DNase* I (Fermentas, Lithuania) set to a final volume of 50 µl. Reaction was incubated at 37°C for 30 min. followed by incubation at 65°C for 15 min. to denature *DNase* I. The treated RNA was extracted with phenol: chloroform: isoamyl alcohol (25:24:1) by centrifugation at 13,000 g for 10 min. at room temperature. To the aqueous phase, 20 µl 3 M sodium acetate and 200 µl isopropanol was added and incubated at room temperature for 10 min. The sample was centrifuged at 13,000 g for 10 min. at room temperature. The RNA pellet was washed with 1 ml of 75% ethanol, centrifuged at 7,500 g for 5 min. and after air-drying was dissolved in 100 µl nuclease free water. The quality and integrity of total RNA was checked on 2% agarose gel containing formaldehyde and then electrophoresed using 1x MOPS gel running buffer.

RT-PCR reactions were performed in 25 µl volumes using the one-step RT-PCR kit (Qiagen, Germany) as per the manufacturer's instructions. RT-PCR amplification reaction mixture containing 1X Qiagen one-step RT-PCR reaction buffer, 0.6 µM of each primer, 400 µM dNTPs, and 5 U Qiagen one-step RT-PCR enzyme mix. Total RNA isolated from leaves of PEG treated coconut plantlets and also control plantlets were used as templates to detect the induction of genes induced during water stress. The housekeeping gene, *rbcL*, was used as reference gene in the experiment and was amplified with the primers rbcLF (5′-ACTGATATCTTGGCAGCATTCC-3′) and rbcLR (5′-TCCATTTGCTAGCTTCACGGA-3′). Reverse transcription was performed at 50°C for 30 min. followed by a denaturation step at 95°C for 15 min., 30 cycles of 94°C for 30 sec., annealing at 52°C for 30 sec., 72°C for 1 min. and finally 72°C for 10 min. After amplification, the amplicons were resolved in 1.2% agarose gel using 1X TBE buffer, stained with ethidium bromide, visualized in gel documentation system and results documented. The sizes of the amplified fragments were estimated by comparison with a 100 bp ladder.

The PCR fragments were excised from the agarose gel and purified using a gel cleanup kit and the eluted DNA fragments were stored at -20°C. Cloning of the eluted fragments was carried out using the InsTA clone™ PCR product cloning kit (Fermentas, Lithuania). After transformation, the cells were finally plated on pre-warmed LB-agar plates [ampicillin (100 ppm)/IPTG (100 ppm)/X-gal (160 ppm)] and incubated overnight at 37°C. The recombinant clones were identified by blue/white colony selection. Colony PCR was carried out for direct analysis of the positive transformants. One colony was picked up and resuspended in 20 µl of the PCR mixture. The reaction mixture was incubated for 5 min. at 94°C to lyse the cells and inactivate the nucleases. PCR amplification was carried out and the products were visualized by agarose gel electrophoresis. Highly pure plasmid DNA was obtained by means of GenElute™ plasmid miniprep kit (Sigma, St. Louis, USA). PCR was carried out using the recombinant plasmid DNA as template. The amplicons were analyzed on 1.5% agarose gel. The plasmids harboring the inserts were then sequenced.

Sequence similarity study was performed by tBLASTX program. A six frame translation was performed with standard genetic code to find out the deduced amino acids for coconut MAPK cDNAs with ORF finder (http://www.ncbi.nlm.nih.gov/projects/gorf/). The frame with maximum length and maximum sequence similarity in tBLASTX search was selected. Motif analysis was performed using online server program PROSITE. Multiple sequence alignment was performed using ClustalW program. The phylogenetic tree was generated by neighbour-joining method by ClustalW and viewed with TreeView. Subcellular localization was estimated using WoLFPSORT (http://wolfpsort.org/) and hydropathicity analysis was analyzed by ProtScale program (http://www.expasy.ch/tools/protscale.html). Secondary structure was predicted by SOPMA program (http://npsa-pbil.ibcp.fr/cgi-bin/secpred_sopma.pl).

Protein structure homology modeling was done with the MODELLER software. Function assignments were made based on the structural homologues and similar homologues were identified for the test protein.

RESULTS AND DISCUSSION

Unfavourable environmental conditions greatly affect plant growth, development and productivity. To survive and adapt to these unfavourable conditions, plants have developed a complex signaling network that senses and protects them from an ever-changing environment. Numerous studies have shown that MAPK cascades play crucial roles in plant responses to multiple stresses (Zhang and Klessig, 24). Analyses of complete genome sequences in *Arabidopsis* and rice have revealed the existence of more than 20 MAPK genes (MAPK group, 9) and 17 MAPK genes in rice (Reyna and Yang, 13), suggesting the complexity of MAPK cascade in plants. A large number of MAP kinases, induced in response to abiotic stresses, have been isolated and characterized from different plant species. Several of these have been shown to be involved in the transmission of water stress signals. In the present study, we report cloning a cDNA sequence encoding a MAPK gene from coconut leaves, *CnMAPK1*, induced by water stress with PEG treatment, using RT-PCR based cloning strategy.

A pair of oligonucleotide primers was designed based on conserved regions of known plant MAPK genes, *viz.*, COMAPKF1:5′-GAGATCAAGCTTCTTAGGCA-3′ (Fig. 1) and COMAPKR1: 5′-GAACATGCAACCCACTGACC-3′ (Fig. 2). The designed primers amplified a 467 bp cDNA fragment through from leaves of coconut plantlets in which water stress had been induced after treatment with 20% PEG (Fig. 3). The MAPK gene was not induced in control plantlets suggesting that the MAPK cascade generally functions in response to water stress in coconut. In an earlier study using coconut calli, a ~46 kDa MAPK like protein was activated shortly after elicitation by treatment with chitosan (Lizama-Uc *et al*., 8), which also activated defence-related transcripts including cDNAs encoding MAPKs. BLAST search (tBLASTX) showed that the isolated cDNA fragment, designated *CnMAPK1* (GenBank accession No. FJ200378) showed high sequence homology with many known plant MAPKs, *viz*., *MPK7* and *MPK5* from *Zea mays*, *SIPK* and *MAPK6* from *Oryza sativa* and *FLRS* from *Triticum aestivum* (93% identity).

CnMAPK1 had one open reading frame of 409 bp and encoded a putative polypeptide of 136 amino acids. Alignment of the deduced protein sequence with MAPKs from other plants indicated that CsNMAPK contained conserved subdomains that were characteristic of serine/threonine protein kinases (Hirt, 4). The TEY motif, which includes the threonine and tyrosine

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                                GAGATCAAGCTTCTTCGGCA
Nicotiana_benthamiana       CTTTGAGAGAGATCAAGCTTCTTCGGCATATGGATCATGAAAACATTGTT 360
Nicotiana_attenuata         CTTTGAGAGAGATCAAGCTTCTTCGGCATATGGATCATGAAAACATTGTT 449
Lycopersicon_esculentum     CTTTGAGAGAGATCAAGCTTCTTCGACATATGGATCATGAAAATATTGTT 389
Solanum_tuberosum           CTTTGAGAGAGATCAAGCTTCTTCGACATATGGATCATGAAAACATTGTT 412
Capsicum_chinense           CTTTGAGAGAGATCAAGCTTCTTCGGCATATGGATCATGAAAACATTGTT 156
Papaver_rhoeas              CTCTACGCGAGATTAAGCTTCTTCGCCATATGGATCATGAAAACGTAGTG 550
Triticum_aestivum           CGCTGCGGGAGATCAAGCTGCTCCGCCACATGGACCACGAGAATATTGTT 366
                            *  *  * ***** ***** ** ** ** ***** ** ** **  * **
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Fig. 1. Conserved region selected for design of forward primer.

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                                                GAACATGCAACCCACTGACC
Nicotiana_benthamiana       CTATACTGCAGCAATTGATGTATGGTCAGTGGGTTGCATTTTCATGGAAT 760
Nicotiana_attenuata         CTATACTGCTGCAATTGATGTATGGTCAGTGGGTTGCATTTTCATGGAAT 849
Lycopersicon_esculentum     CTATACTGCAGCAATTGATGTATGGTCAGTGGGTTGCATCTTCATGGAGT 789
Solanum_tuberosum           CTATACTGCAGCAATTGATGTATGGTCAGTGGGTTGCATCTTCATGGAGT 812
Capsicum_chinense           CTATACTGCAGCAATTGATGTTTGGTCAGTCGGTTGCATGTTC------- 549
Papaver_rhoeas              ATATACTGCTGCTATTGATGTGTGGTCTGTAGGCTGCATTTTCATGGAAT 950
Triticum_aestivum           ATACACTGCAGCAATTGATGTGTGGTCTGTGGGCTGTATATTTATGGAAC 766
                             ** ***** ** ******** ***** ** ** ** ** **
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Fig. 2. Reverse complement of conserved region selected for design of reverse primer.

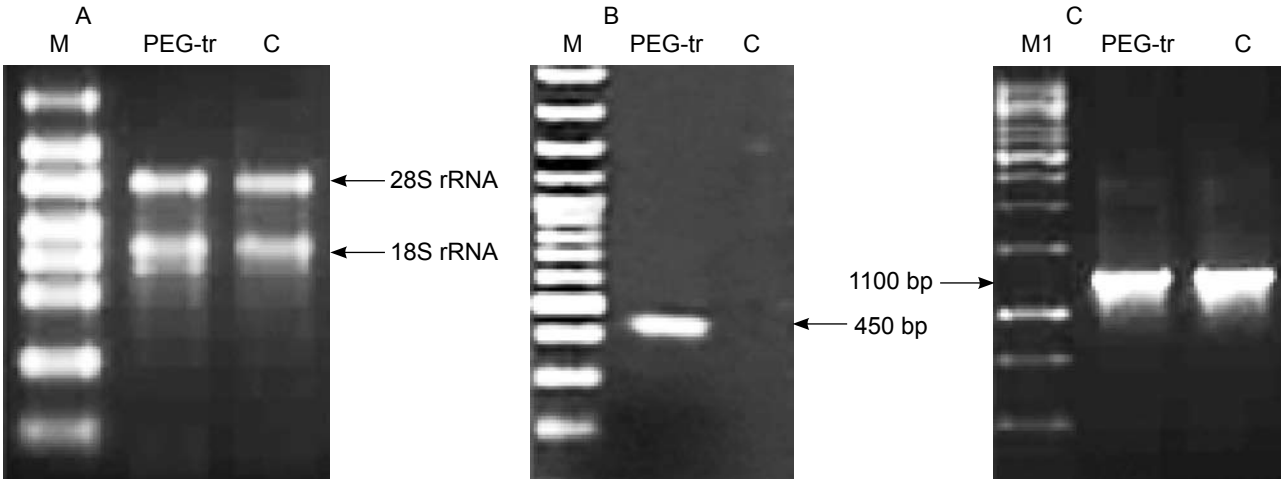

Fig. 3. (A) RNA isolation (B) RT-PCR amplification of coconut transcript for MAPK (C) RT-PCR amplification of coconut transcript for rbcL.
M: 100 bp DNA ladder; M1: 1 kb DNA ladder; PEG-tr: 20% PEG treated; C: Control.

CnMAPK1 -FAIRDIIPPSVWDTYNDVYIAYELMDTDLHQIIRSNQALSEEHCQYFLYQILRGLKYIH 63
TaFLRS IVAIRDIIPPAQRTAFNDVYIAYELMDTDLHQIIRSNQALSEEHCQYFLYQILRGLKYIH 180
PcMAPK6 VVAIRDIIPPPQREAFDDVYIAYELMDTDLHQIIRSNQGLSEEHCQYFLYQILRGLKYIH 175
BnMAPK3 IIAIRDVVPPPLRREFSDVYIATELMDTDLHQIIRSNQGLSEEHCQYFLYQLLRGLKYIH 156
AsMAP1 IVGLRDVIPPSIPQSFNDVYIATELMDTDLHHIIRSNQELSEEHCQYFLYQLLRGLKYIH 154

VLHRDLKPSNLLL KICDFG TEY

CnMAPK1 SANVLHRDLKPSNLLLNANCDLKICDFGLARTTSETDFMTEYVVTRWYRAPELLLNSSEY 123
TaFLRS SANVLHRDLKPSNLLLNANCDLKICDFGLARTTSETDFMTEYVVTRWYRAPELLLNSSEY 240
PcMAPK6 SANVLHRDLKPSNLLLNANCDLKICDFGLARITSETDFMTEYVVTRWYRAPELLLNSADY 235
BnMAPK3 SAKVIHRDLKPSNLLLNANCDLKICDFGLARPTSENEFMTEYVVTRWYRAPELLLNSSDY 216
AsMAP1 SANVIHRDLKPSNLLLNANCDLKICDFGLARPSSESDMMTEYVVTRWYRAPELLLNSTDY 214

CnMAPK1 TAAIDVWSVGCNV---------- 136
TaFLRS TAAIDVWSVGCIFMELMDRKPLFPGRDHVHQLRLLMEVFPCGISSLQLIGTPNEADLDFV 300
PcMAPK6 TAAIDVWSVGCIFMELMNRQPLFPGRDHVHQLRLLT----------ELIGTPTEADLGFV 285
BnMAPK3 TAAIDVWSVGCIFMELMNRKPLFPGKDHVHQMRLLT----------ELLGTPTESDLGFT 266
AsMAP1 SAAIDVWSVGCIFMELINRAPLFPGRDHMHQMRLIT----------EVIGTPTDDDLGFI 264

Fig. 4. Alignment of predicted CnMAPK1 protein with MAKPs from *Triticum aestivum* (TaFLRS), *Petroselinum crispum* (MAPK6), *Brassica napus* (BnMAPK3) and *Avena sativa* (AsMAP1). The conserved sub-domains of protein kinases have been indicated.

residues whose phosphorylation is necessary for MAP kinase activation and is a feature of MAPK, was also conserved (Fig. 4). Construction of a dendrogram with deduced amino acids after removal of primer regions showed that CnMAPK1 closely related to *Triticum aestivum* MAPK (Fig. 5). MAPK cascades usually comprise three protein kinases. The activation of serine/threonine MAPK occurs via phosphorylation of conserved threonine and tyrosine residues in the catalytic sub-domain VII and VIII (TEY) and is affected by dual specific MAPK kinases (MAPKKs), which in turn are activated by serine/ threonine MAPKK kinases (MAPKKKs). Based on the predicted amino acid sequence homology and phosphorylation motif, plant MAPKs can be divided into at least four groups (A-D) (Ligterink and Hirt, 7), and it has been assumed that MAPKs within one group have similar functions among plant species (Jonak *et al*., 5). Phylogenetic analysis of CnMAPK1 with other homologous sequences revealed that CnMAPK1 belongs to group A.

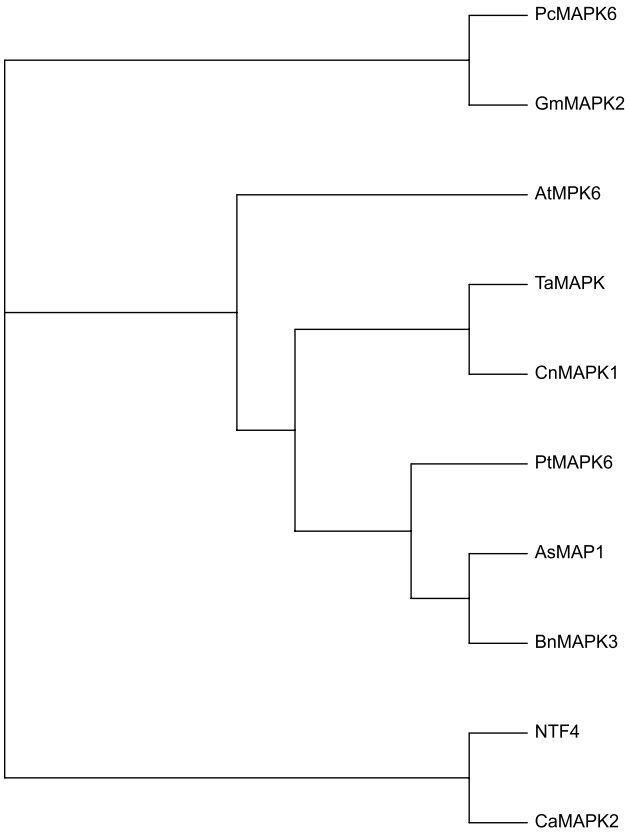

Fig. 5. Phylogenetic tree of primer regions removed CnMAPK1 with other plant MAPKs. The protein sequences of the MAPKs used for construction of the tree are listed in the GenBank database under the following accession numbers: PcMAPK6 (25052802), GmMAPK2 (33340593), AtMPK6 (15224359), TaFLRS (27542952), PtMAPK6 (254047134), CnMAPK1 (209486624), AsMAP1 (871984), BnMAPK3 (54402040), NTF4 (2499615) and CaMAPK2 (121551091).

Hydropathicity analysis with ProtScale programme showed that hydrophilic amino acids were distributed over the peptide symmetrical indicating CnMAPK1 to be a hydrophilic protein (Fig. 6). SOPMA analysis revealed that overall folding of CnMAPK1 is basically built with α-helices (46.32%) and random coils (30.88%) that are connected with extended strands (16.18%) and β-turns (6.62%) (Fig. 7). WoLFPSORT prediction tool of protein localization sites predicted CnMAPK1 to be localized to the cytoplasm, matching well with cytoplasmic location and function of MAPKs (Wilson *et al.*, 14). Transmembrane analysis by TMHMM software displayed that there were no transmembrane domains in CnMAPK1.

Homology modelling provided further support to the cloning strategy. Structure similar to CnMAPK1 was taken from PDB via the local alignment search tool (BLASTP) found in NCBI. Human mitogen-activated protein kinase (PDB Id: 2ZOQ) gave the maximum identity (E value of $3e^{-46}$). The selected template structure and the target sequence were aligned. The pairwise alignment of template and target sequence gave 63% identity overall. The motif region found in CnMAPK1 was predicted with PROSITE. A protein kinase domain region of VLHRDLKPSNLLL obtained via PROSITE was selected for the homology modelling. The assuming structure, developed through the MODELLER, resulted in a spatial architecture of CnMAPK1 very similar to human ERK-2 (Fig. 8).

These findings provide a starting point for elucidating the function of MAPK in water stress induced plant signaling pathway in coconut.

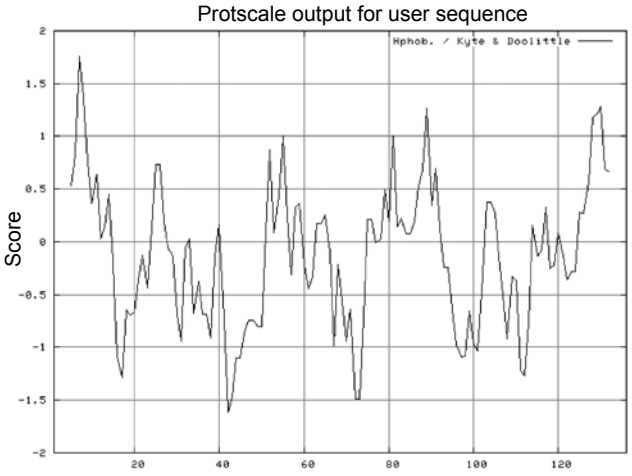

Fig. 6. Hydropathy analysis of predicted coconut CnMAPK1 protein based on Kyte and Doolittle values. Values below zero are negative and hydrophilic.

Fig. 7. The secondary structure of CnMAPK1. Alpha helix, extended strand, beta turn and random coil are indicated, respectively, with the longest, the second largest, the second shortest and the shortest vertical lines.

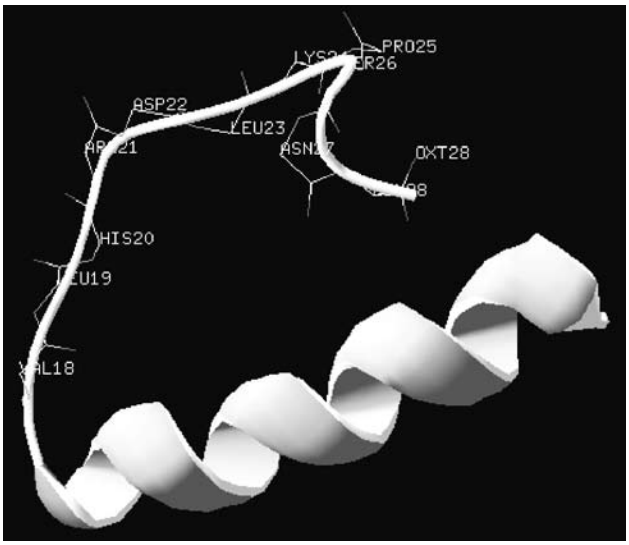

Fig. 8. Predicted 3-D structure of coconut CnMAPK1 protein generated using MODELLER program.

ACKNOWLEDGEMENT

This work was supported by a grant from Department of Biotechnology, Govt. of India New Delhi.

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Received: February, 2011; Revised: December, 2011; Accepted: March, 2012