Chapter 3

Molecular cloning and functional characterization of \textit{AhMPK3}, a mitogen-activated protein kinase gene of Peanut \textit{(Arachis hypogaea L.)}
Chapter 3

3.1 Introduction and Background

3.1.1 Peanut

The genus *Arachis* is native to South America, probably from a region including Central Brazil and Paraguay (Gregory and Krapovickas 1980). There are 69 species described in the genus, assembled into nine sections, according to the morphology, geographic distribution and crossability (Krapovickas and Gregory 1994). Some of these species have been used for forage in South America, but the most important species is the cultivated peanut, *Arachis hypogaea* L. This crop is widely grown in more than 80 countries in the Americas, Asia and Africa (Singh and Singh 1992). The origin of the genus *Arachis*, its taxonomy, cytogenetics and genomes relationships, the botanical classification, and reproductive development have been extensively covered in a review article by Holbrook and Stalker (2003).

Cultivated peanut (*Arachis hypogaea* L) is one of the world’s most important oilseed crop, along side soybean, cottonseed, rapeseed, and sunflower. It is also a rich source of edible oil and vegetable protein and an important crop for both human and animal food. Peanut is the second most important grain legume crop worldwide after soybean, with a production of 33 million tons in 2003/04 (FAO 2003). Soybean and peanut provide more than 35% of the world’s processed vegetable oil. Peanut is produced throughout the tropics and warmer regions of the subtropics, but is particularly important in Africa, Asia and in the United States (FAO 2003). The cultivated peanut (*Arachis hypogaea* L.) is an allotetraploid, with two genomes, classified as AA and BB, according to cytogenetic characters. Similar genomes to those of *A. hypogaea* are found in the wild diploid species of section *Arachis*, which is one of the nine *Arachis* sections.

**Peanut genomics**

With the completion of the *Arabidopsis*, rice and poplar whole genome sequencing projects, a vast amount of valuable data has been generated to facilitate cross-species genome comparison in the plant kingdom. The peanut genome size is significantly larger (2,800 Mb) than the currently sequenced plants (Temsch and Greilhuber 2000), such as
Arabidopsis (128 Mb), rice (420 Mb), and Medicago (500 Mb) (Guo et al. 2008). It is practically unrealistic to completely sequence the whole peanut genome in the near future. The EST projects of peanut have started couple of years back only and by 2007 dbEST release (032307), the number of Arachis ESTs deposited in genbank database were a meager 19,790 (Guo et al. 2008), in sharp contrast to the large number of ESTs in the database of the top five plant species including Arabidopsis (1,276,131), rice (1,211,154), maize (1,161,193), wheat (855,272) and barley (437,728). This clearly shows that peanut genomics is at a very early stage and the identification and isolation of genes of peanut and their characterization will greatly help in increasing its genomic resources and understanding of the plant development and response to various environmental cues.

3.1.2 Plant MAPKs

For living cells to respond to the external stimuli or environment and adapt to them, it is necessary to transmit the signal from outside the cell to inside in a coordinated manner, and finally to the nucleus, where the required gene expression takes place. Living in inevitably changing environmental conditions, plants should respond to them in the most befitting manner in order to survive against the odds. For this purpose they have evolved a variety of signal transduction mechanisms, which transduce the perceived external signal to the inner cellular components for appropriate response to combat such pressures. At the molecular level, the perception of extracellular stimuli and the subsequent activation of defense responses require a complex interplay of signaling cascades, in which reversible protein phosphorylation plays a central role (Yang et al. 1997). Activation and de-activation of enzymes through phosphorylation / de-phosphorylation by kinases and phosphatases allows for fast and specific signal transduction. One particular signal transduction mechanism, the MAP kinase cascade, plays an important role in many different eukaryotic organisms, from yeast, through Dictyostelium, Drosophila and Caenorhabditis to mammals, and also plants. Mitogen-activated protein kinases (MAPKs) comprise a family of serine/threonine protein kinases highly conserved among eukaryotes, which mediate intracellular phosphorylation events.
linking receptor activation to the control of cell proliferation, chemotaxis, differentiation and stress response (Schaeffer and Weber 1999).

Mitogen-activated protein kinase (MAPK) cascade is a conserved transduction mechanism involving three functionally related components (Widmann et al. 1999). The upstream MAPKKKs (MAP kinase kinase kinases), which phosphorylate and activate the downstream MAPKKs (MAP kinase kinases), which in turn phosphorylate and activate the MAPKs (MAP kinases). Phosphorylation targets of activated MAP kinases include both nuclear and cytosolic proteins (Morris 2001). The mammalian MAPKs have been classified into three subgroups based on the phylogeny and function (Kultz 1998). The first subgroup is referred to as extracellular signal-regulated kinases, which are involved in differentiation and cell cycle regulation. The MAPKs in this subgroup are characterized by the specific dual phosphorylation motif TEY (Seger and Krebs 1995). The other two subgroups are the stress-activated protein kinase/Jun N-terminal kinase subfamily, in which TPY is the phosphorylation motif, and the p38/HOG1 subfamily, which uses TGY as the phosphorylation site (reviewed by Canman and Kastan 1996; Kyriakis and Avruch 1996).

Numerous protein kinases, with close sequence similarity to mammalian MAPKs, have been identified in plants (reviewed by Stone and Walker 1995; Hirt 1997; Mizoguchi et al. 1997; Tena et al. 2001; Zhang and Klessig 2001; Agarwal et al. 2003). Most plant MAPKs are associated with the subgroup of extracellular signal-regulated kinases based on phylogeny (Kultz 1998). Several plant MAPKs has been identified, which are activated in response to pathogens (Ligterink et al. 1997; Zhang and Klessig 1997, 1998b; He et al. 1999), cold (Jonak et al. 1996), salinity (Mikolajczyk et al. 2000), drought (Jonak et al. 1996), and wounding (Seo et al. 1995, 1999; Bögre et al. 1997; Zhang and Klessig 1998a; He et al. 1999). Plant MAPKs can also be activated by fungal elicitors (Suzuki and Shinshi 1995), hormones like salicylic acid (Zhang and Klessig 1997), and abscisic acid (Knetsch et al. 1996; Burnett et al. 2000), jasmonates and ethylene (Schweighofer and Meskiene 2008). Apart from this, several MAPKKs (Morris et al. 1997; Hackett et al. 1998; Hardin and Wolniak 1998; Ichimura et al. 1998a; Kiegerl et al. 2000; Yang et al. 2001; Xu et al. 2008) and MAPKKKs (Ichimura et al. 1998b;
Kovtun et al. 2000; Frye et al. 2001; Zhang et al. 2007) have been cloned and characterized.

Mitogen-activated protein kinase (MAPK) pathways in plants have been implicated in signal transduction for a wide variety of stress responses (Jonak et al. 2002; Colcombet et al. 2008; Menges et al. 2008). In the monocot model plant rice, several MAPKs were characterized to be involved in both biotic and abiotic stress responses (Agrawal et al. 2003; Cheong et al. 2003; Reyna et al. 2006; Roshila and Yang 2007; Lee et al. 2008). For most of the rice MAPKs only expression data are available with few exceptions of functional characterization (Roshila and Yang 2007) like OsBWMK1 whose ectopic expression in tobacco resulted in constitutive PR gene expression and enhanced resistance to fungal (Phytophthora parasitica) and bacterial (Pseudomonas syringae) infection (Cheong et al. 2003). RNAi (RNA interference) of OsMAPK5 was shown to induce constitutive PR gene expression and enhanced resistance to fungal (Magnaporthe grisea) and bacterial (Burkholderia glumae) infection (Xiong and Yang 2003). OsMAPK5 was found to positively regulate drought, salt, and cold tolerance and negatively modulate PR gene expression and broad-spectrum disease resistance. And recently, OsMPK6 was shown to negatively regulate rice disease resistance to bacterial pathogens (Yuan et al. 2007).

Based on the completed Arabidopsis genome sequence, 20 MAPKs, 10 MAPKKs and 60 MAPKKKs were identified; they were divided into four groups (A-D). MAPKs belonging to groups A, B and C all possess a TEY motif in their activation loop, while members of group D harbor a TDY motif (MAPK group 2002). Group A MAPKs have been most frequently found to be involved in environmental and hormonal responses. AtMPK3 and AtMPK6 and their apparent orthologs in other species are present in group A and are activated by many environmental stresses (MAPK group 2002). Group B MAPKs have been less well studied, but appear to be involved in both environmental stress responses and cell division. Extensively studied MAPK of group B is AtMPK4, which was shown to be activated in response to abiotic stresses, bacterial elicitor flagellin and harpin (Teige et al. 2004; Suarez-Rodriguez et al. 2007). Loss of function mutants concluded that AtMPK4 functions as a negative regulator of defense responses. Recently Qiu et al. (2008) demonstrated the in vivo interactions of AtMPK4 responsible for its
negative regulatory role in defense. Information on the Group C MPKs is limited, although microarray analysis detected circadian-rhythm-regulated expression of MPK7 (Schaffer et al. 2001). Recent reports suggest that group C MAPKs also play a role in stress responses (Maisa et al. 2008; Zong et al. 2008). Group D MPKs, which include eight members of the Arabidopsis MPKs, are notable for the TDY motif in their T-loop and their extended C-terminal region relative to Groups A, B and C. Group D MPKs also lack the C-terminal CD domain, which is consistently found in members of the other MPK groups. Group D is not studied extensively and members were found to be involved in biotic stress responses like, rice BWMK1 and alfalfa TDY1 are induced by blast fungus and wounding (He et al. 1999; Schoenbeck et al. 1999; Reyna et al. 2006) respectively.

AtMPK3, AtMPK6 and their apparent orthologs in other species are present in group A and are found to be activated by many environmental stresses and shown to be involved in non-host resistance (Zhang and Klessig 1998a; Zhang et al. 2000), gene for gene signal transduction (Zhang and Klessig 1998b; Romeis et al. 1999), hypersensitive response (Liu et al. 2003; Stulemeijer et al. 2007), wounding (Seo et al. 1995), response to elicitors (Zhang et al. 2000; Daxberger et al. 2007), and several abiotic stresses (Jonak et al. 1996; 2004; Samuel et al. 2000; Ahlfors et al. 2004). Recent studies showed their involvement in phytoalexin biosynthesis (Ren et al. 2008), response to herbivores (Wu et al. 2007), key regulators of stomatal development and patterning (Wang et al. 2007), anther (Hord et al. 2008) and ovule (Wang et al. 2008) development.

AtMPK3 orthologs, NtWIPK (Nicotiana tabaccum), LeMPK3 (Lycopersicon esculentum) and MsMMK4 (Medicago sativa) were very well studied and were found to be induced in response to various biotic and abiotic elicitors (Mizoguchi et al. 1996; Jonak et al. 1996; 2004; Bogre et al. 1997; Zhang et al. 2000; Holley et al. 2003; Mayrose et al. 2004; Wan et al. 2004.). In tobacco, mechanical wounding induced rapid transcript accumulation and activation of WIPK (wound-induced protein kinase, Seo et al. 1995). Transgenic plants overexpressing NtWIPK showed constitutive PI-II transcript accumulation, WIPK activity, and higher jasmonic acid (JA) levels compared to wild type (Seo et al. 1999). JA quantification in NtWIPK silenced plants demonstrated that NtWIPK is involved in the production of wound induced JA (Seo et al. 2007). Several orthologs of AtMPK3 were shown to play a crucial role in plant defense responses like
over-expression of MK1, which encodes the Capsicum ortholog of NtWIPK, display elevated JA levels and resistance to blast fungus in transgenic rice plants (Lee et al. 2004). Plants overexpressing TIPK (Trichoderma-Induced MAPK), an AtMPK3/NtWIPK ortholog from cucumber were more resistant to pathogenic bacterial attack than control plants (Shoresh et al. 2006). And the suppression of NtWIPK or its orthologs led to increased susceptibility against pathogens as well (Sharma et al. 2003; Shoresh et al. 2006). For example AtMPK3 mutant plants exhibit reduced camalexin accumulation after B. cinerea infection in Arabidopsis (Ren et al. 2008). Although the involvement of AtMPK3 and its orthologs was well established in wound and systemin signaling responses (Seo et al. 1995; Holley et al. 2003), their role in plant response to herbivore attack was not well explored till recently. Kandoth et al. (2007) and Wu et al. (2007) demonstrated that LeMPK3 and NaWIPK are involved in regulating the defense response against herbivore attack in L. esculentum and N. attenuata respectively. Although several AtMPK3/NtWIPK orthologs from various plants were overexpressed and shown to confer resistance to microbial pathogens, there were no reports on the performance of plants against herbivore attack.
3.1.3 Objectives of the present work:

Though peanut is one of the widely cultivated oilseed crops with economical and nutritional importance, extensive genomic information is not available in public databases pertaining to it. The availability of genetic information would enhance the understanding of mechanisms involved in plant development and stress responses (Guo et al. 2008). This has been accomplished to some extent by the completion of some of the peanut EST projects (Luo et al. 2005a; Guo et al. 2008). Considering the lack of extensive genomic information of peanut and the significant role of MAPKs in regulating plant defense and stress responses, the following objectives were framed.

1. Isolation of partial cDNA corresponding to MAPKs of peanut (Arachis hypogaea L.) using degenerate primers.
2. Obtaining corresponding full length cDNA using RACE.
4. Expression analysis in response to various cues.
5. Functional characterization of peanut MAPK by heterologous expression in tobacco.
3.2 Materials and Methods

3.2.1 Plant material
Detached leaves of peanut (Arachis hypogaea cv. JL-24) from 2-3 week old plants grown in the greenhouse were used in all experiments. Tobacco (Nicotiana tabacum cv. Xanthi) seeds were surface sterilized with 4% sodium hypochlorite for 10 min, washed 4-5 times with sterile distilled water and allowed to germinate on Murashige and Skoog (MS) medium (Murashige and Skoog 1962). Individual germinated seedlings were transferred to culture bottles with MS medium and maintained aseptically.

3.2.2 Treatment with chemicals and abiotic stresses
Compound leaves (quadrifoliate) detached from peanut plants were kept in a tray with a moist filter paper saturated with sterile distilled water and covered with a polythene bag to maintain humidity and left overnight to stabilize the wound signal. For various chemical treatments, leaves were floated in the corresponding solution. The treatments given were 500 µM salicylic acid (SA), 100 µM methyl jasmonate (MeJA), 100 µM abscisic acid (ABA), 25 mM hydrogen peroxide (H$_2$O$_2$), 200 mM mannitol, 100 mM sodium chloride (NaCl), 100 µM sodium nitroprusside (SNP) and the treatment with water served as control. Wounding was performed by damaging the leaf lamina with a sharp blade and a pointed forceps and cold treatment was given by shifting the leaves to a cold chamber (4°C). Samples were collected at regular intervals, quickly frozen in liquid nitrogen, and stored at -80°C until use. Mannitol, NaCl and H$_2$O$_2$ are obtained from Himedia, India. Rest of the chemicals used for treatments were purchased from Sigma-Aldrich, USA.

3.2.3 DNA and RNA isolation
Leaves of peanut and tobacco were frozen in liquid nitrogen and ground into a fine powder. Total Genomic DNA was then extracted by the cetyltrimethylammonium bromide (CTAB) procedure (Murray and Thompson, 1980). RNA was isolated from
samples harvested at various intervals using TRI reagent (Sigma-Aldrich, USA) following the manufacturers instructions. The quality and concentration of RNA and DNA samples were examined by ethidium bromide-stained agarose gel electrophoresis and spectrophotometric analysis.

3.2.4 RT-PCR and amplification of Partial cDNA and cloning of PCR products
Reverse transcription reaction was performed using 4 µg of total RNA, 500 ng of Oligo-dT primer and 2 µl of 10mM dNTP, 2 µl of 10X RT buffer and 1 µl (200 units) of reverse transcriptase enzyme (Sigma Aldrich, USA) in a 20 µl reaction at 37°C for 60 min followed by 15 min at 75°C for heat inactivation of the enzyme. One-tenth volume of the RT reaction mixture was used as a template for PCR using degenerate primers, IntF as the sense primer and IntR as the antisense primer (Table 3.1). PCR was performed in a volume of 25 µl containing 200µM dNTP mix, 2mM MgCl2 0.8 pmol each of forward and reverse primer, 1X PCR buffer and 1.25 units of Taq polymerase (Invitrogen, USA). A gradient PCR was performed with varying annealing temperatures with cycling parameters of 94°C for 3min for initial denaturation followed by 33 cycles of 94°C for 1 min (denaturation), 51,5°C /53°C /54,4°C /56°C for 1 min (annealing) and 72°C for 1min (extension), a final extension at 72°C was performed for 10 min in an Eppendorf Thermal Cycler.

3.2.5 Cloning of PCR products
All the PCR amplified products were electrophoresed, gel eluted (Gel cleanup kit, Eppendorf, Germany) and ligated into cloning vector pTZ57R/T (Insta clone T/A cloning kit, Fermentas, Germany). The ligation reactions were transformed into Escherichia coli (DH5α) host cells and the bacterial colonies carrying the inserts were identified by blue/white selection and subsequently confirmed with colony PCR.

3.2.6 DNA sequencing and Sequence analysis
For all the clones, both DNA strands were completely sequenced on an automated DNA sequencer commercially. The sequence similarity search was performed using BLASTN and BLASTP at NCBI website (www.ncbi.nlm.nih.gov). Nucleotide translations were
performed using (DNA/RNA to protein) Translate tool at ExPASy. (www.expasy.ch). Sequence alignments were done using CLUSTALW multiple sequence alignment tool at European Bioinformatics Institute (www.ebi.ac.uk). Phylogenetic analysis was performed using CLC Free Workbench (http://www.clcbio.com). Reverse complementation and other sequence formatting were done using BCM search launcher (www.searchlauncher.bcm.tmc.edu). The promoter sequences were analyzed using the PLACE database (http://www.dna.affrc.go.jp/htdocs/PLACE/).

3.2.7 Isolation of full length cDNA of \textit{AhMPK3}

To obtain the full length sequence of \textit{AhMPK3}, 5' and 3' Rapid amplification of cDNA ends (RACE) reactions were performed using 5'/3' RACE kit (Roche applied sciences, Germany) following the manufacturer’s instructions with minor modifications. Based on the available partial cDNA sequence, primers were designed for 5' and 3' RACE. The list of gene specific primers and their sequences were given in Table 3.1. For 5' RACE, the first strand cDNA was synthesized in a 20 µl reaction containing cDNA synthesis buffer, dNTP mix, 6 µg of total RNA, 12.5 μM gene specific primer, and Transcriptor reverse transcriptase at 55°C for 60 minutes, followed by 85°C for 5 minutes to heat inactivate the enzyme. Instead of using a gene specific primer, a degenerate primer WyrR (Table 3.1) designed against conserved (WYRAPE) amino acids of subdomain VIII of MAPKs was used in reverse transcription. To add a homopolymeric tail, the purified cDNA was incubated with dATP in the presence of terminal transferase at 37°C for 30 minutes, followed by heat inactivation of enzyme at 70°C for 10 min. The dA-tailed cDNA was used as a template to amplify the 5' region using oligo dT-anchor primer (Table 3.1) and gene specific primer Ah443R in a 50µl reaction with 1.5mM MgCl₂, 200uM dNTP (Invitrogen), 1X PCR buffer, and 2.5 units of \textit{Taq} DNA polymerase (Invitrogen). The cycling conditions were 94°C for 3 min, followed by 34 cycles of 94°C for 45 sec, 55°C for 45 sec, 72°C for 1 min and a final extension of 10 min at 72°C. The PCR product obtained was diluted and used as template in nested PCR using Ah270R and PCR anchor primer (Table 3.1) following similar cycling conditions. For 3' RACE, the cDNA synthesis reaction conditions were the same as in 5' RACE, except that an OligodT-Anchor primer was used, instead of Gene specific or degenerate primer. One twentieth of
the cDNA was used in a PCR reaction of 50 µl, amplified with PCR anchor primer and gene specific primer AH3P1F. PCR conditions were 94°C for 3 min followed by 35 cycles of 94°C for 30 sec, 54°C for 30 sec and 72°C for 1 min 30 sec with a final extension of 10 min at 72°C. The PCR product was diluted and used in the nested PCR using nested primer Ah3P2F in combination with PCR anchor primer. The full length cDNA of AhMPK3 was deduced by aligning 5’ and 3’ RACE product sequences with partial cDNA. The full length cDNA, including the 5’ and 3’ UTR was amplified using gene specific primers AhMK31F and AhMK31497R. The PCR reaction was performed in a 50µl volume containing 2.0mM MgCl2, 200µM dNTP (Invitrogen), 1X PCR buffer, and 2.5 units of Taq DNA polymerase (Invitrogen). The cycling conditions were 94°C for 3 min, followed by 34 cycles of 94°C for 45 sec, 55°C for 45 sec, 72°C for 3 min. and a final extension of 15 min at 72°C.

3.2.8 Isolation of the AhMPK3 genomic clone
Two gene specific primers AhMK31F and AhMK31497R were used to PCR amplify the AhMPK3 gene using Arachis hypogaea genomic DNA as template. PCR was carried out using 100ng of genomic DNA in a 50 µl reaction volume containing 2.0 mM MgCl2, 200 µM dNTP (Invitrogen), 1X PCR buffer, and 2.5 units of Taq DNA polymerase (Invitrogen). The PCR conditions were 94°C for 3 min, followed by 34 cycles of 94°C for 45 sec, 55°C for 45 sec, 72°C for 3 min. and a final extension of 15 min at 72°C.

3.2.9 Genomic Southern blot analysis
Peanut genomic DNA (20 µg) was digested with Bell, EcoRI, EcoRV, HindIII and XbaI (Fermentas, Germany) respectively, fractionated on 0.8% agarose gel and visualized by ethidium bromide staining. The DNA from the gel was transferred onto a Hybond N+ membrane (Amersham Pharmacia, UK) through capillary transfer and the blot was UV-cross-linked. A 281 bp fragment of the 3′ UTR was amplified using primers AhMK31216F and AhMK31497R and labeled with [α-32P] dATP using Primer-a-Gene® Labeling System (Promega, USA) according to the manufacturer’s Instruction. The membrane was pre-hybridized for 3-4 h at 65 °C and hybridized for 16–18 h at 65 °C using [α-32P] labeled probe. Following hybridization, the membrane was washed with 2 X SSC, 0.1% SDS and 1 X SSC, 0.1% SDS for 10 min and 0.1X SSC and 0.1% SDS for 5
min each respectively at 65 °C and then exposed to an X-ray film (Kodak, Japan) using two intensifying screens at −80 °C.

3.2.10 Semi-quantitative RT-PCR.
Reverse transcription was performed as described earlier in Material and Methods except that instead of 4 µg, only 2 µg of RNA was used. The amount of cDNA and number of cycles for linear increase of PCR products was determined (data not shown). Conditions, which consistently gave product in linear range, were used for all experiments. The expression of \textit{AhMPK3} in peanut was studied using specific primers for amplifying the entire coding region, ORF-F and ORF-R (Table 3.1). Gene specific primers were employed for expression analysis of the defense related genes in transgenic and wild type (WT) tobacco plants (Table 3.3). The house-keeping gene actin amplified using primers Act-F and Act-R served as the internal control. The amplified products were analyzed on 1.2% agarose gel and visualized by staining with ethidium bromide.

3.2.11 Localization of AhMPK3
\textit{AhMPK3} cDNA was amplified from reverse-transcribed RNA using Primers ORF-F2 and ORF-R (Table 3.1) engineered with \textit{SmaI} and \textit{BamHI} restriction sites respectively. The resulting fragment was cloned into pEGAD vector (Cutler et al. 2000) digested with appropriate restriction enzymes to make an in-frame fusion with GFP to obtain pEGAD: \textit{AhMPK3}. The pEGAD control vector and pEGAD: \textit{AhMPK3} constructs were mobilized into \textit{A. tumefaciens} strain EHA105 by freeze thaw method (Holsters et al. 1978). The resulting strains were utilized in transient transformation of tobacco leaves by agroinfiltration as described by Yang et al. (2000). In brief, agrobacterial strains harboring the corresponding clones were grown overnight at 28°C in the presence of appropriate antibiotics, pelleted at 3000g for 5 min and diluted to an OD\textsubscript{600} of 1.0 in 10 mM MES pH 5.6, 10 mM MgCl\textsubscript{2}, 150 µM acetosyringone and infiltrated into the leaves using a needle less syringe. After 48 h, GFP was visualized with a laser scanning confocal microscope (Leica). 10mM H\textsubscript{2}O\textsubscript{2} was infiltrated into the leaves 60min before observation to study the dynamic localization of \textit{AhMPK3} in response to oxidative stress. Water was used for mock infiltration.
3.2.12 Development of Transgenic tobacco plants

The complete open reading frame of \textit{AhMPK3} was amplified using primers ORF-F and ORF-R (Table 3.1) cloned into pTZ57R vector, and confirmed by sequencing. \textit{NcoI} and \textit{BamHI} restriction sites were incorporated in the primers at 5’ and 3’ ends to facilitate cloning into plant expression vector pRT100 by digesting it with the same set of enzymes such that the coding region will be flanked by 35S promoter and Poly A signal in the sense orientation. The entire cassettes with \textit{AhMPK3} coding region flanked by 35S promoter and Poly A signal was released from pRT100 by digesting with \textit{HindIII} and cloned into binary vector pRD400 digested with \textit{HindIII}. The recombinant binary vector was mobilized into \textit{Agrobacterium tumefaciens} EHA105 using freeze thaw method. Transgenic tobacco plants were generated by standard leaf disc transformation method (Horsch et al. 1985). Leaf discs (0.5 cm in diameter) were cut out from leaves of tobacco plants grown aseptically and the leaf discs were agroinfected by soaking them in agrobacterium suspension for 4-5 min. \textit{Agrobacterium}-infected leaf discs were cultivated on MS medium with 2 mg l\(^{-1}\) BAP and 0.1 mg l\(^{-1}\) NAA at 28°C for 2 d, and then transferred to the MS medium containing 2 mg l\(^{-1}\) BAP and 0.1 mg l\(^{-1}\) NAA supplemented with 150 mg l\(^{-1}\) kanamycin and 250 mg l\(^{-1}\) cefataxime. Control uninfected leaf discs were kept directly on selection medium with kanamycin. The explants were subcultured to fresh selection medium every 15 days. Shoots obtained were rooted on MS medium containing 100 mg l\(^{-1}\) kanamycin and 250 mg l\(^{-1}\) cefataxime. The rooted shoots were transferred to soil, acclimatized at 28°C and shifted to greenhouse.

3.2.13 Molecular analysis of transgenics

DNA was extracted from 5-6 week old T\(_0\) transgenics, and around 100ng of DNA was used for PCR amplifications. Putative transgenics were confirmed by amplifying the genomic DNA with 35SF (Table 3.1) as the sense primer designed against the CaMV35S promoter region and AhMPK3 ORF-R as the antisense primer. Southern Analysis for transgenic plants was performed as described earlier in Materials and Methods, except that the genomic DNA was digested with \textit{EcoRI} and hybridization was done using \([\alpha^{32}\text{P}]\) dATP labeled \textit{nptII} fragment obtained by the amplification of neomycin
phosphotransferase gene with nptII F and nptII R primers. For Northern analysis of transgenic plants, 20 µg of total RNA was fractionated on a 1.2% agarose-formaldehyde gel. Equal loading and RNA integrity was checked by ethidium bromide staining, and fractionated RNA was transferred by capillary action overnight to a Hybond-N+ nylon membrane (Amersham Pharmacia, UK) using 20X SSC. The RNA on the membrane was fixed by UV cross-linking. Probe labeling, hybridization, and detection were the same as in the procedure described for southern blot hybridization. \[^{\alpha-32P}dATP\] labeled \(AhMPK3\) ORF was used as probe in Northern hybridization.

### 3.2.14 Herbivore bioassay

Bioassay was performed according to detached leaf method described by Sharma et al. (2005) with minor modifications. In brief, leaves of two month old WT and transgenic plants were cut at their petiole with a sharp blade and immediately planted into 3% agar-agar in a petri dish. Bioassays were conducted with first, second and third instar larvae of the generalist herbivore, \(Spodoptera litura\) with five larvae per leaf and five replications for each sample. The bioassay plates were maintained in a culture room at 28°C ± 1 and a photoperiod of 16:8 (Light/ Dark). The experiments were terminated when > 80% of the leaf area was consumed in WT plants, generally 5 days for first instar, 3 days for second instar, and 2 days for third instar larvae respectively. The area of leaf damage and mass of larvae were recorded after each experiment and mean of five replications was plotted. The data were analyzed by ANOVA and student’s \(t\)-test.
3.3 Results

3.3.1 Isolation of full length cDNA of AhMPK3

In an attempt to clone mitogen-activated protein kinases from *Arachis hypogaea*, degenerate primers were designed from two conserved regions of MAP kinases. The forward primer IntF corresponded to the ATP binding motif (GAYG I/VVC) in subdomain I of protein kinases (Hanks et al. 1988) and the reverse primer IntR corresponded to the region including TEY motif (MTEYVVT) present between subdomain VII and VIII. Using RT-PCR, a single fragment of 465 bp was obtained, which might be the product of amplification of several different MAP kinases, because the primers were designed against highly conserved regions. The PCR product was cloned and several clones were sequenced and identical clones were grouped together. Among them two clones were identified as diverse, but closely related and considered as different clones (Fig 3.1). Sequence similarity search using BLANTN and BLASTP showed a high similarity to the existing MAPKs from several plant species. One of the two clones i.e MPK2 (Fig.3.1) was further extended using 5’ and 3’ RACE to obtain the full length cDNA.

By utilizing the sequence information of the partial cDNA fragment, gene specific primers were designed. The same RNA used for amplification of the partial clones was used as a template for reverse transcription using oligo dT- Anchor primer (for 3’ RACE) and degenerate gene specific primer WyrR (for 5’ RACE) followed by the amplification of corresponding 5’ and 3’ cDNA ends with designed nested gene specific primers. By using a degenerate primer for reverse transcription, several members of the gene family would get reverse transcribed. Hence, the same cDNA could be used as template for isolating 5’ regions of different genes of the same family using nested gene specific primers in combination with PCR anchor primer. In 5’ RACE, a 750 bp product was obtained with the gene specific primer Ah443R in combination with PCR anchor primer. The product was further confirmed by amplification with the nested primer Ah270R. In 3’ RACE, a 1100 bp product was obtained with the primer Ah3P1F, which was confirmed with the nested primer Ah3P2F. All the PCR products obtained were cloned and
sequenced. The sequences were aligned to obtain the overlapping regions and the full length cDNA of AhMPK3 was deduced. Based on the sequences of RACE products, two gene specific primers AhMK1 IF and AhMK1 1497R were designed and the full length cDNA was amplified and sequenced, which was identical to the deduced cDNA. AhMPK3 cDNA was submitted to NCBI Genbank database under the accession number DQ068453.

Figure 3.1 Sequences of the two partial clones of MAPK (MPK1 and MPK2) isolated from A. hypogaea using degenerate primers. IntF and IntR are degenerate primers used and the letters in bold indicates the primer annealing region.
45
1 cac taa cct tcc ttc gtc cct ctc aac ggt ttc tag aga gag aga
46 gag aga aac taa aag tct ccg ttt aga gag aga tag aaa aca
91 ccg ATG GCC GGC GTT AAT CCA AAC GGT GCC GCG GAT TTT CCG GCG
135 M A G V N P N A D F P A
136 GTC CGG ACT CAC GGT GAA CAG TTC ATT CAG TAC AAC ATC TTC GGT
180 V P T H G G F I Q Y N I F G
181 AAC CTC TTT GAG GTC ACC GCT AAG TAC CGT CCT CCG ATC ATG CCT
225 N L F E V T A K Y R P P I M P
226 ATC GGT CGT GGA CAC GTC AAC GGT GCC GCG GAT TTT CCG GCG
270 M E G V N P N G A A D F P A
271 GAG ACT AAT GAG CTC TTT GTC GAG AAG ATA GCT AAG GCG TTC
315 G E T N E L V A V K K I A N A F
316 GAT AAT CAC ATG GAT GCC AAG CGC ACA CTC GGT GAC TTT AAG CTC
360 L E H M D A K R T L R F K L
361 CTG AGG CAT CTG GAT CAT GAA AAG ATG AGT GCC TTA AGA GAT GTC
405 N L R H L D H E N I H S A N I I R
406 ATT CCT CCA CCC TTT CTC AGA GAG TTT ATG GTC TAT ATT GCA
450 I P P P L R R E F N D V Y I A
451 ACG GAG CTC ATG AGT ATT ATT ATT CCG TCC GAT
495 G E T N E L D T D H H I I R S N
496 CAG GCC CTG TCG GAG GAA CAC TGC CAG TAC TTC TTG TAT CAG ATT
540 Q G L S E E H C Q Y F L Y Q I
541 TCT CGT GGG CTG AAG TAC ATA CAT TCT GCA AAC ATA ATT CAT AGA
585 L R G L K Y I H S A N I I R
586 GAT TCG AAA CCA AGC AAT CTG TCG TGT TGT AAT GCA AAT TGT GAC TTT
630 L R G L K Y I H S A N I I R
631 AAG ATT ATT GAT TTT GGT CTT CGG CCA CAC TTA GAA AAT GAT
675 E D H M D A K R T L R F K L
676 TTC ATG ACA GAG TAG TGT GCT ACA AGG TGG TAG ACG CCT GCT GAA
720 M E H M D A K R T L R F K L
721 CTG CTG TTT AAG TCT TCG AAG AAG ATG GAG TTT AAC
765 L R G L K Y I H S A N I I R
766 TCT GGT GGT TGC ATC TTT ATG GAA CTC ATG AAT AAA AAG CCT CTC
810 L R G L K Y I H S A N I I R
811 CTC CCA GGG AAG GAT CAC CTG CAT CAG CTG CTA TGT ACA GAG
855 L P G K D H V H Q M R L L T E
856 CTT CTC GAC ACT CCA ACT GAG GCA GAC CTT GGG TTA GTG AAA AGT
900 L L G T P T E A D L G L V K S
901 GAG GAT GCC AGA AGA TAC ATC CGA CAA CTT CCA TAA TAT GCT GCG
945 E D A R R Y I R Q L P Q Y A R
946 CAA CCT TTA GCT AGG ATC TTT CCC CAT GAT CAT CCC TTT GCC ATT
990 L R G L K Y I H S A N I I R
991 Q P L A R I F P H V H P L A I
225
Figure 3.2 Nucleotide and deduced amino acid sequences of AhMPK3. The nucleotides and amino acids are numbered. Asterisk (*) indicate the stop codon. The conserved TEY motif is underlined. The UTR regions are represented by lower case letters.
3.3.2 Nucleotide and protein sequence analysis.
The full length AhMPK3 cDNA is 1514 bp long including the ORF, 5′, 3′ untranslated regions and the poly-A tail (Fig 3.2). Sequence analysis revealed an open reading frame of 1113 bp potentially encoding a 371 amino acid polypeptide. The reading frame shown was the only possible reading frame in the cDNA and had both the translational initiation codon ATG at nucleotide 94 and translational stop codon TAA at nucleotide 1207. A 93 bp of 5′ untranslated region and a 292 bp 3′ untranslated region followed by poly-A tail were present flanking the open reading frame. A potential polyadenylation signal (AATAAA) was found in the 3′ UTR at1249bp. Threonine and Tyrosine amino acids of TEY motif were present at 197 and 199 positions respectively. The 371 amino acids encoded protein had a predicted molecular mass of 42615.98 Da and a calculated isoelectric point (pI) of 5.52 (Compute pI/MW tool, ExPASy).

3.3.3 Multiple sequence alignment and phylogenetic analysis
Sequence alignment of the predicted amino acid residues of AhMPK3 with closely related MAP kinases indicated that it contained all the eleven conserved subdomains of protein kinases described previously (Hanks et al. 1988) and possessed a dual phosphorylation activation motif (TEY) located between subdomains VII and VIII (Fig. 3.3). The phylogenetic analysis showed that AhMPK3 belongs to the A1 subgroup of MAPK family (Fig. 3.4) (MAPK group 2002). The AhMPK3 protein exhibited 94% sequence identity to GmMPK1 from Glycine max, 91% to MsMMK4 from Medicago sativa, 85% to CsTIPK and PtMPK3-1 of Cucumis sativus and Populus trichocarpa respectively. Most well characterized A1 subgroup members of MAPK family, AtMPK3 (Arabidopsis thaliana), NtWIPK (Nicotiana tabacum) and LeMPK3 (Lycopersicon esculentum) shared 81% similarity with AhMPK3 protein.
**Figure 3.3** Alignment of deduced amino acid sequences of AhMPK3 with closely related MAPKs from other plant species. The eleven subdomains of protein kinases are marked with roman numerals. Threonine (T) and Tyrosine(Y) residues whose phosphorylation is required for MAPK activation are indicated by Asterisk. At: *Arabidopsis thaliana*, Nt: *Nicotiana tabacum*, Ms: *Medicago sativa*, Cs: *Cucumis sativus*.
Figure 3.4 The phylogenetic relationship of AhMPK3 with other MAPK family members from different plant species. A phylogenetic tree based on the genetic distance of the protein sequences was constructed using ClustalW program and CLC-free workbench 3.1. The MAPK members used for construction of the tree are listed in the GenBank database under the following accession numbers: AhMPK3(DQ068453); AtMPK1(NM_100895); AtMPK2(NM_202320); AtMPK3(NM_114433); AtMPK4(NM_116367); AtMPK5(AK176361); AtMPK6(NM_129941); AtMPK7(NM_127374); AtMPK8(NM_179354); AtMPK9(NM_112686); AtMPK10(NM_115841); AtMPK12(NM_130170); AtMPK13(NM_001035913); AtMPK14(NM_119808); AtMPK15(NM_106026); AtMPK16(NM_121906); AtMPK17(NM_126206); AtMPK18(NM_104229); AtMPK20(NM_129849); CsTIPK(DQ118734); GmMPK1(AF104247); GmMPK2(AF329506);
LeMPK1 (AY261512); LeMPK3 (AY261514); MsMMK2 (X82268); MsMMK3 (AJ224336); MsMMK4 (X82270); MsMSK7 (X66469); NtWIPK (D61377); NtSIPK (U94192); OsBWMK1 (AF177392); OsMAPK5 (AF479883); OsMAPK6 (AJ535841); Oswjumk1 (AJ512643); OsRMAPK2 (AF194416); OsMAPK4 (AJ251330); PtMPK3-1 (estExt_fgenesh4_pm.C_LG_IX0462); PtMPK6-1 (estExt_fgenesh4_pm.C_LG_VII0025); ZmMPK4 (AB016801); ZmMPK5 (AB016802); Ah: Arachis hypogaea, At: Arabidopsis thaliana, Cs: Cucumis sativus, Gm: Glycine max, Le: Lycopersicon esculentum, Ms: Medicago sativa, Nt: Nicotiana tabacum, Os: Oryza sativa, Pt: Populus trichocarpa, Zm: Zea mays.

3.3.4 Genomic and structural organization of AhMPK3 gene
The copy number of AhMPK3 gene was analyzed by Southern blot analysis. Arachis hypogaea genomic DNA was digested with restriction enzymes BclI, EcoRI, EcoRV, HindIII, XbaI and subjected to hybridization using a 281 bp 3' UTR region of AhMPK3 as a probe. This 281 bp region did not harbor restriction sites of any of the above enzymes used. Two distinct bands were detected in samples digested with EcoRI, EcoRV and HindIII (Fig. 3.5). This can be explained by the fact that peanut (Arachis hypogaea) is an amphidiploid, which carries two sets of diploid chromosomes. Hence, one band corresponds to the AhMPK3 gene and the second band in the southern blot presumably belonged to its ortholog in the second genome of peanut. The sample digested with BclI showed three distinct bands, which could be explained by the possibility of BclI site in the AhMPK3 ortholog in the second genome of peanut. The single band detected in XbaI digested sample could be due to the near equal size of two bands which co-migrated giving the appearance of a single band. The simple hybridization pattern suggested that gene encoding AhMPK3 might exist as a single copy in peanut genome (Fig. 3.5).
The genomic clone of AhMPK3 was amplified using gene specific primers designed against 5’ and 3’ termini of full length AhMPK3 transcript using peanut genomic DNA as template. A 3036 bp fragment was obtained which was cloned and sequenced. The genomic structure of the AhMPK3 gene was established by the alignment with the corresponding cDNA, which revealed that coding region of AhMPK3 contained six exons and five introns (Fig. 3.6). The size of introns varied from 104bp (III intron) to 810bp (II intron). All the 5’ and 3’ splice junctions follow the typical, canonical consensus di-nucleotide sequence GT-AG (Table 3.2). All the introns are A + T-rich; in particular they present an elevated T content (Table 3.2), which is a peculiar feature of many plant introns (Ko et al. 1998).

AhMPK3 gene structure was compared with its orthologs from Arabidopsis (AtMPK3) and poplar (PtMPK3-1 &3-2) (Fig. 3.6), where PtMPK3-2 is presumed paralog of PtMPK3-1 (Nicole et al. 2006). Comparative analysis of exon-intron junctions in all the three species indicate that the numbers of exons, and their sizes as well as the intron phases were extremely well conserved. Whereas the intron lengths were varied

Figure 3.5 Genomic organization of AhMPK3. 1- BclI, 2- EcoRI, 3- EcoRV, 4- HindIII, 5- XbaI. Arachis hypogaea genomic DNA was digested with the indicated restriction enzymes, fractionated on 0.8% agarose gel, blotted on to a nylon membrane and hybridized with AhMPK3- 3’UTR fragment as a probe.
among the species, poplar and peanut introns were much longer than the corresponding Arabidopsis introns (Fig. 3.6). AhMPK3 genomic clone can be accessed from NCBI GenBank under the accession number EU182580.

**Figure 3.6** Graphical representation of AhMPK3 gene structure and its comparison with its orthologs from poplar (PtMPK3-1&3-2) and Arabidopsis (AtMPK3). Exons are represented by closed boxes and introns by dark lines, the dotted lines represent the 5' and 3' UTRs respectively. The individual exons, introns and UTRs length were given in base pairs. Numbers between brackets correspond to the intron phase. Drawings are not exactly to scale. NA: Not available. PtMPK3-1&3-2 genomic sequences were retrieved from DOE Joint Genome Institute database (http://genome.jgi-psf.org/Potr1_1/Potr1_1.home.html) and AtMPK3 genomic sequence was obtained from The Arabidopsis Information Resource (http://www.arabidopsis.org).
3.3.5 Expression analysis of *AhMPK3*

To determine the expression pattern of *AhMPK3* in response to various stress treatments, a semi-quantitative RT-PCR was carried out using RNA samples harvested from the materials frozen at various intervals (Fig. 3.7). The results showed that a basal level of *AhMPK3* is maintained in leaves, which got upregulated upon the incidence of stress. The difference observed in transcript levels at 0 h in various treatments could be due to the plant physiological differences or Circadian rhythms. In response to wounding, *AhMPK3* transcript expression reached to peak in 15 min, which gradually came down to the basal level by 6 h. Since both the pathogen and wound stress lead to H$_2$O$_2$ accumulation in plants, we studied the effect of H$_2$O$_2$ on the expression pattern of *AhMPK3*. With H$_2$O$_2$ application, *AhMPK3* got upregulated gradually up to 30 min after treatment followed by a sudden decline at 60 min and gradual rebound by 12 h. In response to salicylic acid and methyl jasmonate, which are the signaling molecules for SAR and wound signaling respectively, the gene expression showed an upregulation during the later stages of the treatment. To examine the influence of nitric oxide (NO), which is an emerging essential component of plant defense, treatment with SNP which is a NO donor caused steady state increase in the *AhMPK3* transcript reaching a peak by 12 h. The analysis of *AhMPK3* transcript in response to mannitol, which causes osmotic stress, showed a gradual increase by 30 min, and declined to basal level by 24 h. ABA, which is the major signaling molecule for abiotic stress responses, induced *AhMPK3* transcript accumulation at 30 min and a gradual decline before rebounding at 24 h after treatment. NaCl treatment had no significant impact on the expression pattern of *AhMPK3* (data not shown). Treatment with water served as control and it showed a slight increase in *AhMPK3* transcripts after 15 min. This suggested that the increase observed at 15 min in various chemical treatments was a combined effect of placing the leaves in an aqueous solution and its corresponding chemical.
Figure 3.7 Expression analysis of *AhMPK3* in response to various treatments using semi-quantitative RT-PCR. A. wounding, B. hydrogen peroxide (H$_2$O$_2$), C. methyl jasmonate (MeJA), D. salicylic acid (SA), E. Sodium nitroprusside (SNP), F. abscisic acid (ABA), G. mannitol, H. cold and I. water (H$_2$O). The semi-quantitative RT-PCR reactions of *AhMPK3* are performed as described in materials and methods. cDNA synthesized from RNA samples collected at specific intervals of different treatments were amplified using gene specific primers *AhMPK3* (ORF-F and ORF-R). Actin, which served as an internal control, was amplified using Act F and Act R primers.

3.3.6 Subcellular localization of *AhMPK3*

Studies in mammals and yeast have shown that stimulus-induced activation of MAPKs correlated with dynamic changes in their localization, whereby the proteins often translocated to, and accumulated in, the nucleus of the cell. This is often required due to the nuclear localization of key MAPK substrates, including transcription factors involved in the control of gene expression (Brunet et al. 1999). Localization of *AhMPK3* was analyzed by constructing an N-terminal GFP fusion and transiently expressing in tobacco.
leaves using agroinfiltration. As previous studies showed that AtMPK6/NtSIPK and AtMPK3/NtWIPK were activated by hydrogen peroxide and superoxide (Kovtun et al. 2000; Samuel et al. 2000; Moon et al. 2003), we studied the dynamic changes in the localization of AhMPK3 in response to H$_2$O$_2$. Under untreated conditions, AhMPK3 localized simultaneously in nucleus and cytoplasm. Upon treatment with H$_2$O$_2$ the staining intensity and frequency of nuclear staining further increased in a majority of cells observed, showing predominant nuclear localization (Fig. 3.8). Cells expressing GFP from control vector alone showed GFP in the entire cell and was unaffected by water or H$_2$O$_2$ treatments.

Figure 3.8 Subcellular localization of AhMPK3. Control vector pEGAD and pEGAD:AhMPK3 were transiently transformed to *Nicotiana tabacum* leaves through agroinfiltration. GFP was visualized in epidermal cells using Confocal laser scanning microscope 48 h post agroinfiltration. Water (H$_2$O) or 10mM hydrogen peroxide (H$_2$O$_2$) were infiltrated 1 h before GFP visualization. (Continued…)
A. pEGAD control vector shows expression of GFP throughout the cell with out any treatment or when treated with H₂O or H₂O₂.

B. pEGAD: AhMPK3 shows GFP localized to both cytoplasm and nucleus with out any treatment or upon treatment with H₂O

C. pEGAD: AhMPK3 shows predominant nuclear localization of GFP upon treatment with H₂O₂. (Bar 20µm)

3.3.7 Generation of transgenic plants and Herbivore resistance assay

Transgenic tobacco plants with *AhMPK3* under CaMV35S promoter were raised using *Agrobacterium* mediated leaf disc transformation (Horsch et al. 1985). T₀ transgenic plants were confirmed by genomic PCR (Fig 3.9A) and Southern hybridization (Fig 3.9B). Northern analysis confirmed the *AhMPK3* expression in T₀ transgenic plants (Figure 3.9C). Two single copy, high transgene expression lines (T-5, T-8) and a moderately expressing line (T-9) derived from the corresponding progeny of the primary transgenic plants by selfing were selected for further analysis in T₂ generation. T₂ seeds were germinated on half strength MS medium supplemented with 100mg/l kanamycin. The kanamycin tolerant plants were selected and transferred to soil in green house along with WT plants germinated on half strength MS medium.
Figure 3.9 A. PCR confirmations of T₀ transgenic plants. Genomic DNA was isolated from wild type and T₀ transgenic plants and used as template. A fragment of 1530bp was amplified using promoter and gene specific primers (35SF and AhMPK3 ORF-R). That indicated both the presence of the transgene and the correct promoter–transgene fusion/orientation.

B. Southern analysis of T₀ transgenic plants. Genomic DNA of wild type (WT) and T₀ transgenic plants (1-9) was digested with EcoRI and electrophoresed, blotted and hybridized with $[^{32}P]$-labelled nptII gene as probe.

C. Northern analysis of T₀ transgenic plants. Total RNA was prepared from wild type (wt) and T₀ transgenic plants (1-10). RNA samples (20μg) were separated by denaturing formaldehyde-agarose gel electrophoresis, blotted, and hybridized with $[^{32}P]$-labelled AhMPK3 probe. Ethidium bromide stained ribosomal RNA bands are shown as loading controls.
All the T₂ transgenic plants were first confirmed with genomic PCR (data not shown) followed by northern hybridization to analyze the expression of *AhMPK3* in T₂ transgenic plants (Fig.3.10). As we performed all the hybridization and washing steps at high stringency conditions, we did not observe any signal due to cross reactivity in wild type plants using AhMPK3 as probe. Herbivore resistance of transgenic plants against the common cut worm *Spodoptera litura* was examined by the level of leaf damage and gain of larval weight upon feeding on leaves of two month old WT and transgenic plants. All the transgenic plants showed a high level of resistance to the first instar larvae, a moderate resistance towards second instar and low resistance towards third instar larvae respectively (Fig. 3.11; Fig. 3.12A). Analysis of larval weights after feeding showed that the final biomass of larvae fed on WT plants was significantly higher compared to the larvae fed on high expression transgenic lines (Fig.3.12B).

**Figure 3.10** Northern analysis of T₂ transgenic plants for *AhMPK3* expression. Total RNA was prepared from WT and T₂ transgenic plants (T-5, T-8 and T-9). RNA samples (20 µg) were separated by denaturing formaldehyde-agarose gel electrophoresis, blotted, and hybridized with α-³²P-labelled *AhMPK3* probe. Ethidium bromide stained ribosomal RNA bands are shown as loading controls.
**Figure 3.11** Representative pictures of leaf damage in wild type (WT) and transgenic plants (T-5, T-8 and T-9) after feeding by first instar larvae of *S. litura* for five days.

**Figure 3.12**

A. Leaf area consumed (cm²) in WT and transgenic plants with first, second and third instars of *S. litura* larvae. Data are mean values ±SE and asterisks indicate significant difference between WT and transgenic plants. (* indicates $P<0.05$)

B. Mean mass (±SE) of individual *S. litura* larvae after feeding on Wild type (WT) and transgenic plants (T-5, T-8, T-9). Asterisks indicate significant difference between WT and transgenic plants. (* indicates $P<0.05$)
3.3.8 Transcript levels of defense response genes in transgenic plants.

Transgenic plants showing enhanced resistance to Spodoptera litura were analyzed for the levels of various defense related transcripts using semi-quantitative RT-PCR. Transgenic plants displayed constitutively higher levels of lipoxygenase1 (LOX1), pathogenesis related proteins PR1a, PR1b, acidic β-1,3-glucanase, acidic chitinase, protease inhibitor II (PI-II) and ornithine decarboxylase (ODC) transcripts compared to WT plants (Fig. 3.13). Transcript levels of isochorismate synthase (ICS), lipoxygenase 3 (LOX3), and 1-aminocyclopropane-1-carboxylic acid synthase (ACS3a) which are the key enzymes in salicylic acid (SA), jasmonic acid (JA) and ethylene biosynthesis respectively, were unaffected. Apart from these, other wound or JA responsive gene transcripts like protease inhibitor I (PI-I), allene oxide synthase (AOS), allene oxide cyclase (AOC) were almost similar in both WT and transgenic plants (Fig. 3.13). Transcripts levels of basic PR5 (osmotin) and defensin, which are known to be regulated by ethylene and JA synergistically were also unaffected. AhMPK3 transgenics also exhibited slightly higher transcript levels of tobacco native MPK3 i.e NtWIPK (Fig. 3.13).

To study the effect of AhMPK3 overexpression on wound induced defense responses, we analyzed the level of PI-II transcripts upon wounding in WT and transgenic plants. Upon wounding, transgenic plants accumulated PI-II rapidly to high level by one hour and maintained it throughout the study (Fig. 3.14), whereas the WT plants exhibited a gradual increase of PI-II transcripts in a time dependent manner but not to the level exhibited by the transgenic plants. PR1b, which is a wound inducible pathogenesis related protein, also displayed a similar rapid induction in transgenic plants (Fig.3.14). LOX3, a key regulator of wound induced JA biosynthesis, was induced rapidly in both WT and transgenic plants reaching peak level by 1 h after wounding. In WT plants, LOX3 transcripts reached the basal level in a time dependent manner, but the transgenic plants maintained higher LOX3 transcript levels even 24 h after wounding. Neither the constitutive accumulation of defense related transcripts nor the rapid accumulation of PI-II transcripts upon wounding was observed in aged transgenic plants of five months or older (data not shown).
Figure 3.13 Transcript profile of defense responsive genes in WT and Transgenic plants (T-8). Data for line T-8 alone is provided here. The experiments were performed on all other lines with similar results. Semi-quantitative RT-PCR was performed using total RNA of WT and transgenic plants. AOS: allene oxide synthase, AOC: allene oxide cyclase, LOX: lipoxygenase, PI: Protease inhibitor, PR: pathogenesis related protein, ICS: isochorismate synthase, ODC: ornithine decarboxylase.
Figure 3.14 Time course analysis of wound induced expression of LOX3, PR1b and PI-II in WT and transgenic plants (T-8). Data for line T-8 alone is provided here. The experiments were performed on all other lines with similar results. Semi-quantitative RT-PCR was performed using total RNA extracted from samples collected at the indicated time intervals of WT and transgenic plants after wounding. LOX: lipoxygenase, PR: Pathogenesis related protein, PI: Protease inhibitor.

3.3.9 In silico analysis of promoter regions
A previous study by Yap et al. (2005) identified NtWIF (N. tabacum WIPK interacting-factor) as a downstream target of NtWIPK in tobacco plants. Based on overexpression and suppression of NtWIF in transgenic tobacco plants, Chung and Sano (2007) demonstrated that NtWIF regulates the wound-responsive genes containing the core sequence of auxin-responsive element (ARE). AhMPK3, being an ortholog of NtWIPK, probably might share common downstream targets of NtWIPK, when expressed in tobacco plants. With increased transcript levels of PR1b, acidic β-1, 3-glucanase, acidic chitinase in AhMPK3 transgenics, it was tempting to speculate that NtWIF might be working downstream of AhMPK3 in transgenic tobacco plants.

As a positive feedback regulation was reported in case of NtWIPK and NtWIF interaction (Chung and Sano 2007), we tried to ascertain the possible role of NtWIF in the current transgenic plants by analyzing the transcripts levels of NtWIPK.. The higher
transcript levels of NtWIPK further demonstrated the possible role of NtWIF in regulating gene expression in AhMPK3 transgenic plants (Fig. 3.13). Acidic β-1,3-glucanase, acidic chitinase and NtWIPK were already shown to harbor ARE motifs in their promoter regions (Chung and Sano 2007). Hence, a preliminary in silico analysis was carried out for other genes like NtPI-II, NtPR1a, NtLOXI and NtODC-I, which showed differential regulation in AhMPK3 transgenic plants and their upstream regions are available to identify if any of them harbor ARE motif (TGTCTC) in their promoter regions. An ARE motif was found in the NtPI-II gene (Z29537) promoter region at -1564 from transcription start site on the negative strand (Fig. 3.15). However, the upstream regions of NtPR1a (~1.5kb, X05959), NtLOXI (~2.2kb, EF397141) and NtODC-I (~2.0kb, AF233849) did not display any ARE motifs in the available upstream regions, although we can not completely rule out the presence of an ARE motif further upstream in these genes.

Figure 3.15 ARE motif in promoter of NtPI-II. Numbers indicate nucleotide positions upstream to transcription start site (TSS). ARE motif (TGTCTC) is shaded. The NtPI-II coding region only is available in NCBI database (Z29537). Upstream region was taken manually from Balandin et al. (1995).
3.4 Discussion

Being sessile, plants have to defend themselves against a wide range of unfavorable conditions for which they have developed elaborate and complex signaling networks to perceive the signal and respond. Mitogen-activated protein kinase (MAPK) cascade is one such signaling network, which is present in all eukaryotic organisms form yeast to mammals and also in plants. The cascade comprises three classes of hierarchically organized protein kinases, namely MAPKKS, MAPKKs, and MAPKs, which rapidly amplify and transduce extracellular signals into various appropriate intracellular responses (Morris 2001).

A full length cDNA corresponding to a mitogen-activated protein kinase (MAPK) gene from peanut was cloned and based on its high homology with Arabidopsis AtMPK3, the present cDNA was designated as AhMPK3. AhMPK3 contains TEY motif in its activation loop and belongs to the A1 subgroup of MAPK family (MAPK group 2002). AhMPK3 protein shows very high homology with A1 subgroup members from other plants like GmMPK1 (Glycine max), MsMMK4 (Medicago sativa), AtMPK3 (Arabidopsis thaliana), NtWIPK (Nicotiana tabacum). Southern blot analysis revealed that AhMPK3 might exist as a single copy gene in peanut genome and analyzing its genomic clone showed it contains six exons and five introns. Structural organization of AhMPK3 when compared with AtMPK3 (Arabidopsis, a herbaceous plant) and PtMPK3 (Poplar, a woody plant) revealed that the number of exons and introns, exon length and intron phases are well conserved, whereas the intron lengths and length of UTRs varied. This highlighted the conservation of these signaling molecules across various species and a strong negative selection for any alteration in protein sequence (Nicole et al. 2006).

Transcriptional regulation offers an important level of control in plants. Hence, analysis of transcriptional regulation of MAPK cascade components in a given plant species would provide an insight into possible biological functions of these components (Nicole et al. 2006). AhMPK3 orthologs from other plant species were found to be transcriptionally regulated in response to wounding (Seo et al. 1995; Mysore et al. 2004), Systemin and UV light (Holley et al. 2003), cold and drought (Jonak et al. 1996). Like other counterparts of A1 subgroup of MAPK family, AhMPK3 transcripts in peanut were
also induced in response to various cues. In response to wounding, H$_2$O$_2$, NO, mannitol, ABA and cold AhMPK3 exhibited distinct expression. SA and MeJA did not induce significant expression of AhMPK3 at early stage but an upregulation at later stages of the treatments was observed. Previous reports of on TIPK (Shoresh et al. 2006) and LeMPK3 (Mayrose et al. 2004) also suggested that there was no effect of JA on their expression levels.

Identification of subcellular localization of MAPKs would provide an insight into the potential functional roles they harbor in plants. It has long been known that the activation of MAPKs in yeasts and mammals involved their simultaneous transport to the nucleus (Cobb and Goldsmith 2000). The phosphorylated AtMPK3 translocated rapidly to the nucleus upon ozone (O$_3$) exposure (Ahlfors et al. 2004). Elicitation of parsley cell cultures with Pep-13 resulted in the translocation of PcMPK3a/b to the nucleus (Ligerink et al. 1997; Lee et al. 2004). NtWIPK was also shown to simultaneously locate in nucleus and cytoplasm (Yap et al. 2005). Like its homolog NtWIPK, AhMPK3 was also found to localize in both nucleus and cytoplasm. In our experimental system, we utilized the agroinfiltration for transient expression of GFP fusions in tobacco leaves and Agrobacterium itself is known to activate AtMPK3 (Djamei et al. 2007). Although we made observations 48 h after infiltration, we can not completely rule out the possibility of Agrobacterium induced activation and nuclear localization of some portion of AhMPK3 protein. However, AhMPK3 protein predominantly accumulated in the nucleus after H$_2$O$_2$ application, which clearly showed that H$_2$O$_2$ induced activation of AhMPK3 resulted in subsequent translocation to the nucleus. In a recent report, Qui et al. (2008) elegantly demonstrated that WRKY33 was sequestered with MPK4 and MKS1 in the nucleus under normal conditions. But, challenge with Pseudomonas syringae or flagellin lead to the activation of MPK4 and phosphorylation of MKS1 and subsequent release of WRKY33, which activates camalexin synthesis through regulation of PAD3. This provides a new mechanism by which plant MAPKs could also regulate the gene expression by releasing transcription factors in the nucleus upon activation. A study in yeast also suggests that MAPKs may physically associate with promoters and influence the transcription of certain genes (Pokholok et al. 2006). Hence, the nuclear localization of AhMPK3 might have significant implications in gene regulation.
Recent evidence demonstrates the involvement of AtMPK3/NtWIPK orthologs and AtMPK6/NtSIPK orthologs in regulating plant defense response against herbivores using VIGS (Wu et al. 2007; Kandoth et al. 2007). Co-silencing LeMPK1 and LeMPK2 orthologs of AtMPK6/NtSIPK compromised pro-systemin mediated resistance to Manduca sexta herbivory (Kandoth et al. 2007). However, no direct experimental data was available in case of plants overexpressing or silenced AtMPK3/NtWIPK or its orthologs in terms of the effect of their expression conferring resistance against chewing insects. Hence, the transgenic tobacco plants ectopically expressing AhMPK3 were studied for their resistance against Spodoptera litura. AhMPK3 transgenic plants showed enhanced resistance to the attack by the first instar larvae and moderate resistance against second instar larvae. Analyzing the defense response transcripts in transgenic and WT plants showed higher transcript levels of LOX1, PR1a, PR1b, acidic β-1, 3-glucanase, acidic chitinase and PI-II. The transcript levels of isochorismate synthase (ICS) which is a key enzyme in salicylic acid (SA) biosynthesis (Wildermuth et al. 2001; Catinot et al. 2008) were similar in WT and transgenic plants indicating a possible SA-independent upregulation of PR genes in AhMPK3 transgenics.

Except for PI-II and PR1b, whose transcripts were upregulated in transgenics, other genes, which are known to be involved in wound or JA responses like AOS, AOC, LOX3 and PI-I displayed no apparent differences in transcript levels. As PI-II are also regulated by ethylene as well (Balandin et al. 1995; Kim et al. 2003), we studied the transcript levels 1-aminocyclopropane-1-carboxylic acid synthase (ACS3a) involved in ethylene biosynthesis and other ethylene responsive genes like basic PR5 (osmotin) and defensin in transgenic plants, which showed no apparent differences between WT and transgenic plants. The transcript levels of ornithine decarboxylase involved in biosynthesis of nicotine, which is herbivore or wound or JA inducible and ethylene suppressible (Shoji et al. 2000), were higher in transgenic plants compared to WT. This implied a less possible role of ethylene in controlling PI-II levels in transgenic plants.

A time course analysis of PI-II transcripts in response to wounding showed that in WT plants, the accumulation of PI-II transcripts occurred gradually and reached maximum level by 12 to 24 h, whereas the transgenic plants accumulated high levels of PI-II transcripts within 1h, which was maintained through out the time of study. A similar
expression pattern observed for PR1b suggested that other wound induced genes were also induced in a similar way. LOX3 is a wound induced lipoxygenase and LOX3-mediated JA signaling accounts for a major part of induced resistance, when plants are damaged by insect herbivores (Rayapuram and Baldwin 2006). Antisense suppression of LOX3 resulted in herbivore susceptibility indicating a crucial role in herbivore tolerance (Halitschke and Baldwin 2003). Sustained transcript levels of LOX3 in transgenic plants after wounding suggested a better wound induced JA or JA responsive gene induction. Transcript abundance at a given time is an important prerequisite to subsequent production of the corresponding protein required for proper execution of its function. The activation of NtWIPK an ortholog of AhMPK3 was delayed and it requires transcriptional activation and de novo synthesis of a WIPK protein (Zhang et al. 2000). It was postulated that a delayed or lack of activity of WIPK, when treated with phosphatase inhibitors, was likely because of the reduction in upstream kinase activity by the time WIPK accumulated to a significant level (Liu et al. 2003). Like other MAPKs, AhMPK3 also presumably might be activated by its upstream MAPK kinase, which in turn phosphorylates and activates effector proteins that directly or indirectly regulate a spectrum of responses. Hence, by overexpressing AhMPK3, the protein would be available readily to be activated by its upstream kinase upon appropriate signal. In such a case plants overexpressing AhMPK3 would be primed to respond rapidly.

The activities of MAPKs in a cell are controlled by the opposing actions of MAPKKs, which phosphorylate and activate them and MAPK phosphatases, which dephosphorylate and inactivate them (Widmann et al. 1999). The observed constitutive upregulation of defense response genes in transgenic plants could be due to the basal level activity of upstream kinase or the level of corresponding phosphatase is not sufficient enough to inactivate the entire pool of protein in a transgenic plant with high expression levels. It is also possible that more than one MAPKK is involved in the activation of a particular MAPK under different conditions as in case of yeast and animal systems (Widmann et al. 1999; Davis 2000). The higher transcript levels of PR1b, acidic ß-1, 3-glucanase, acidic chitinase and NtWIPK in AhMPK3 transgenics suggested a possible role of NtWIF. In silico analysis of promoter regions of other upregulated genes for possible NtWIF targets identified NtPI-II harboring ARE motif in its promoter region.
Whereas available upstream regions of NtPR1a, NTLOX1 and NtODC-1 did not have ARE motifs. But surprisingly no protease inhibitor gene was reported by Chung and Sano (2007) in their analysis of NtWIF downstream targets. It is not known whether this particular gene was part of their analysis or not. Further experimental evaluation is required to confirm the PI-II regulation by NtWIF. This suggests that genes like acidic β-1, 3-glucanase, acidic chitinase, NtWIPK and PI-II might be regulated by the NtWIF, whereas genes like PR1a, LOX1 and ODC might be under the control of one or more different transcription regulators in AhMPK3 transgenic plants.

For example, AtVIP1 a bZIP type of transcription factor was demonstrated to be regulating AtPR1a expression upon activation of AtMPK3 in Arabidopsis (Djamei et al. 2007). And NaWIPK was found to regulate the transcript levels of MPK4, NaSIPK, WRKY and several CDPKs (Wu et al. 2007). Our results of higher LOX3 and PI-II transcript levels in AhMPK3 transgenic plants upon wounding are in agreement with the VIGS functional analysis of LeMPK3 and NaWIPK, in which silencing of LeMPK3 in 35S:: prosys tomato plants resulted in significant reduction in LoxD transcripts (a homolog of tobacco LOX3) as well as reduced PI-II levels (Kandoth et al. 2007). The silencing of NaWIPK resulted in reduced Trpsin proteinase inhibitor (TPI) activity and reduced LOX3 transcripts with wounding alone and in combination with the Manduca sexta’s oral secretions (OS) application in N. attenuata. This shows that PI-II levels are positively regulated by AhMPK3 or its homologs.

In a recent review, Beckers and Conrath (2007) reported that in Arabidopsis priming by the chemical agent benzo (1,2,3) thiadiazole-7-carbothioic acid S-methyl ester (BTH) is based on enhanced accumulation of mitogen-activated protein kinase 3 (AtMPK3) protein with out displaying MPK3 activity. However, upon exposure to biotic or abiotic stresses, MPK3 enzyme activity was induced to enhanced levels in primed plants, which was associated with boosted defense gene activation and stress resistance. The physiological state, in which plants are able to faster or better activate defense responses, or both, is called the primed state of the plant (Beckers and Conrath 2007). Due to the overexpression of AhMPK3, the transgenic plants presumably are in a primed state, which resulted in rapid induction of PI-II upon wounding. Lack of these enhanced levels of defense response transcripts observed in older AhMPK3 transgenics could be
imputed to the unavailability of downstream regulatory molecules, or due to the absence of active physiological environment in aged plants. Constitutively higher level of various defense gene transcripts as well as rapid induction of protease inhibitor II (PI-II) transcripts upon wounding, which encodes the antidigestive protein and functions as a direct defense against herbivore, might have collectively resulted in the resistance of \textit{AhMPK3} transgenic tobacco against \textit{Spodoptera litura}. The observed transcript levels of various genes in WT and transgenics indicate that regulation is independent of hormones as all the subset of genes known to be regulated by specific hormone were not affected. Sustained levels of \textit{LOX3} in wounded transgenic plants suggested the possible role of \textit{AhMPK3} in regulating stress induced hormone levels. This indicates that \textit{AhMPK3} probably activates transcription factors with well-defined downstream targets.

Essentially, most of the studies on priming in response to wounding and or herbivore attack were associated with use or involvement of plant derived cues like volatile organic compounds (VOCs) that are emitted in response to herbivory (Frost et al. 2008). In the present investigation, we demonstrated the wound induced priming of defense responses in tobacco plants ectopically expressing \textit{AhMPK3} of peanut. The regulatory molecules connecting \textit{AhMPK3} and gene expression are being currently investigated. Our results substantiate the function of \textit{AtMPK3}/\textit{NiWIPK} orthologs in defense against herbivore attack in plants.
Table 3.1 Sequences of oligonucleotides used in the study. See text for details

<table>
<thead>
<tr>
<th>Name of the Primer</th>
<th>Primer Sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IntF</td>
<td>GG(A/C)GC(A/T)TA(C/T)GG(A/C)(A/G)T(A/T/G/C)GT(A/T/G/C)TG</td>
</tr>
<tr>
<td>IntR</td>
<td>(A/T/G/C)GT(A/T/G/C)AC(A/T/G/C)AC(A/G)TA(C/T)TC(A/T/G/C)GTC</td>
</tr>
<tr>
<td>WyrR</td>
<td>(C/T)TC (A/T/G/C)GG(A/T/G/C)GC(A/T/G/C)(G/T)(A/G)TA CCA</td>
</tr>
<tr>
<td>Ah443R</td>
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</tr>
<tr>
<td>Ah270R</td>
<td>AGTTCTGGCAGTGTTCCTCCG</td>
</tr>
<tr>
<td>Ah3P1F</td>
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</tr>
<tr>
<td>Ah3P2F</td>
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</tr>
<tr>
<td>AhMK₃1F</td>
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</tr>
<tr>
<td>AhMK₃1216F</td>
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</tr>
<tr>
<td>AhMK₃1497R</td>
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</tr>
<tr>
<td>ORF-F</td>
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</tr>
<tr>
<td>ORF-R</td>
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<tr>
<td>ORF-F2</td>
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<tr>
<td>PCR anchor primer</td>
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<tr>
<td>Oligo d(T) anchor primer</td>
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</tr>
<tr>
<td>Act F</td>
<td>TGGCATCACACTTTCTACAA</td>
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<tr>
<td>Act R</td>
<td>CAACGGAATCTCTCAGCTCC</td>
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<tr>
<td>nptII F</td>
<td>GAGGCTATTCGGCTATGACTG</td>
</tr>
<tr>
<td>nptII R</td>
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<tr>
<td>35SF</td>
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Table 3.2 Properties of *AhMPK3* gene introns. Upper and lower case letters indicate exons and intron regions, respectively. Letters in bold lower case indicate canonical dinucleotide 5’ & 3’ splice sites.

<table>
<thead>
<tr>
<th>Intron No</th>
<th>Intron size (bp)</th>
<th>Splice junction sequences</th>
<th>A+T (%)</th>
<th>T (%)</th>
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<tbody>
<tr>
<td>I</td>
<td>288</td>
<td>CGTTTGgttaact ......tgagCTCGGT</td>
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<tr>
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<td>37</td>
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</table>

Table 3.3 Sequences of gene-specific primers used in RT-PCR for amplification of defense related gene transcripts. See text for descriptions.

<table>
<thead>
<tr>
<th>Oligo Name</th>
<th>Forward</th>
<th>Reverse</th>
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<tr>
<td>PI-I</td>
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<td>AATCCCTTAGACCCACCTGG</td>
</tr>
<tr>
<td>PI-II</td>
<td>GTTAGTTTCGTCGCTCATCT</td>
<td>CTGCTTACAACAGTTAG</td>
</tr>
<tr>
<td>AOS</td>
<td>CTTGGTCTTCCGAAAGGTC</td>
<td>GACGTCAATACCAACCTG</td>
</tr>
<tr>
<td>PR1a</td>
<td>CTCTTTGCTCTACTACCTTC</td>
<td>GCAAGAGCACAATATCCCT</td>
</tr>
<tr>
<td>PR1b</td>
<td>TTAAACCTCACAATGCAG</td>
<td>AGGGTTGCTCTCAAGATC</td>
</tr>
<tr>
<td>LOX1</td>
<td>CACTTCTACTGATTCATCT</td>
<td>CTCTGTGACATTCATCTGA</td>
</tr>
<tr>
<td>LOX2</td>
<td>AAATGACAGAAACTCCAGC</td>
<td>TAGAAACGCTTCGACAATTC</td>
</tr>
<tr>
<td>glucanase</td>
<td>ATGGCTTTATGCAAATAAATGC</td>
<td>AGCATTGAAAGACATTGGTTTCTGG</td>
</tr>
<tr>
<td>Chitinase</td>
<td>CGGAAAGATAGGACAGACGGTAG</td>
<td>ATACGTCCTAGTATCCATAC</td>
</tr>
<tr>
<td>Defensin</td>
<td>GAGGCAGAAGAATTGTAATCT</td>
<td>AAGCCGAAACATTATATACATAC</td>
</tr>
<tr>
<td>PR5</td>
<td>CTGAGATCTTCTTGTCTTCTTCT</td>
<td>ACTTCCAGGCTTCAGCAAGGAAA</td>
</tr>
<tr>
<td>ODC</td>
<td>CCCTTGATTCCTTCCTC</td>
<td>TAAATTACTACAAAAACAAAAATT</td>
</tr>
<tr>
<td>ICS</td>
<td>TGGCATATGACTTTGCTCTGCACAC</td>
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<td>AOC</td>
<td>CTCTCTAGGTCCCTGCTCCTTGA</td>
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<td>ACS3a</td>
<td>ATAGTTATGAGTGAGAGGC</td>
<td>CCCTGTCTTGTCCCTAGTCT</td>
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3.5 Summary
Mitogen-activated protein kinase cascade plays a very important role in plant signal transduction mechanism. A full length cDNA of 1514 bp length, corresponding to a mitogen- activated protein kinase gene was cloned from peanut (Arachis hypogaea). Based on its high homology with Arabidopsis AtMPK3, the cDNA was designated as AhMPK3. It carried an open reading frame of 1113 bp encoding a 371 amino acid polypeptide. AhMPK3 bears TEY motif in its activation loop and belongs to the A1 subgroup of MAPK family. Southern blot analysis revealed that AhMPK3 might exist as a single copy gene in peanut genome and its structural organization revealed well-conserved nature of these signaling components across different species. AhMPK3, when transiently expressed in tobacco leaves was found to localize in both nucleus and cytoplasm. Transgenic tobacco plants ectopically expressing AhMPK3 exhibited enhanced resistance to the first and second instar larvae of Spodoptera litura and constitutively higher transcripts levels of defense response genes like PR1a, PR1b, LOX1, PI-II etc. Apart from this when wounded, transgenic plants accumulated high levels of PI-II and PR1b transcripts rapidly compared to wild type indicating the occurrence of a priming phenomenon.