Chapter 6: Summary and Conclusions
Plants are immobile organisms, capable of perceiving and responding to endogenous and exogenous signals. The discrimination between beneficial or detrimental stimuli has led to evolution of complex and interconnected signaling pathways in plants. Endogenous stimuli are generally derived from stressed, damaged or malfunctioning cells called Danger-Associated Molecular Patterns, DAMPS (Lotze et al. 2007), promote responses in plant cells. Similarly, exogenous stimuli comprising of PAMPs/MAMPs; virulence factors such as toxins (Friesen et al. 2008), enzymes (Belie'n et al. 2006), effector molecules (To'r 2008), and non-microbial or abiotic stress inducers such as toxic compounds, pollutants, UV-B light, injury, or ozone activate a plethora of signal transduction pathways in plants (Hirt 2009). In response to the pathogen attacks, plants have evolved a multilayered innate immune system to resist infection by the majority of pathogenic microorganisms. The plant cell is surrounded by the cell wall matrix, which acts as the primary barrier for would-be pathogens. The pathogens overcoming this barrier are under molecular surveillance by the plant cell receptors that reside at the cell surface, like membrane bound receptor-like kinases (RLKs), that have an extracellular receptor domain and a cytoplasmic kinase domain; for transmission of the signals inside the cytoplasm. These immunogenic and defense signals trigger extensive transcriptional reprogramming of cells to help the plant combat the invading pathogen (Schwessinger and Zipfel 2008). Further, thus receptor-triggered signaling is channeled inside cells and tissues by a hormone response network that allows the plant to fine tune its reaction to the type of pathogen attacking and the prevailing environment (Spoel and Dong 2008). MAPK cascades are an important component in all these defense-signaling cascades, regulating and transmitting these signals in diverse ways (Zhang and Klessig 2001).

*Leguminosae* are second only to *Graminiae*, in terms of production and agrimonic value to mankind. Similar to other cereal crops, diseases and pests are the major constraints to legume production worldwide (Graham and Vance 2003). However not much is known about the mechanisms governing plant-pathogen interactions in legumes, as these mechanisms are mainly explored in model plants like *Arabidopsis*, Tobacco, Tomato and cereals like Rice. Chickpea is an economically important legume, with a worldwide production of 9.1 million tonnes annually. The major setback to worldwide chickpea production is due to the invasion of pathogenic
organisms like *Fusarium oxysporum*, *Ascochyta rabiei* (Pass.) Lab and pod borer (*Helicoverpa armigera*). Among these, *Ascochyta* blight, caused by *Ascochyta rabiei* (Pass.) Lab, is by far the most damaging disease of chickpea, reported to cause entire crop loss, in the susceptible varieties (Singh and Reddy 1990). The early stages of the disease are critical in the establishment of the disease and survival of the plant. However, the host responses and defense mechanisms during the early infective stages of *Ascochyta* blight are not clearly understood till date. The primary objective of this work was to identify and characterize a MAPK gene, previously isolated during the early events of chickpea-*Ascochyta* interaction, and study its role and activity during defense signaling. We decided to study the role of a truncated MAPK gene, isolated using a Suppression Subtractive Hybridization strategy to isolate early genes, during chickpea-*Ascochyta* interaction. The full-length sequence of the truncated gene *CaMPK1*, obtained form a SSH library, was isolated and used for *in silico* analysis with model as well as legume MAPKs. To understand the behavior of *CaMPK1* during *Ascochyta* infection and with respect to defense signaling molecules, the transcript accumulation of the gene was studied. To interpret and correlate the transcript accumulation with corresponding protein activity, the modulation of the recombinant protein was investigated during the initial events of chickpea-*Ascochyta* interaction.

### Isolation of full-length sequence and *in silico* analysis of CaMPK1

To understand and investigate the role of the truncated MAPK clone isolated from a SSH library of early inducible chickpea genes during *Ascochyta* infection, it was imperative for us to isolate its full-length sequence. The full-length sequence isolation using 5' and 3' RACE yielded a cDNA of 1116 Kb encoding a 371 amino acid protein with an estimated molecular weight of 42.37 kDa. The sequence homology search of various genetic databases, using CaMPK1 sequence did not yield any reports of it being isolated during *Ascochyta* infection or other biotic and abiotic conditions in chickpea. Further, there were no previous reports of any chickpea MAPK being isolated in the various databases. The complete lack of genetic and experimental data on the MAPKs, functional in chickpea, led us to study CaMPK1 in the light of information available from its orthologs in model systems like Arabidopsis, Tobacco, Tomato etc. The sequence analysis showed that CaMPK1
contained the eleven conserved domains of plant MAPKs, having the characteristic protein kinase domain, MAPK signature motif, activation loop and ATP binding signature region. The phylogenetic tree, comparing the CaMPK1 with thirty-eight other well-characterized MAPK from diverse groups and plant, clustered CaMPK1 closest to the Group A1 MAPKs. Further sequence analysis showed that AtMPK3 was its closest ortholog in *Arabidopsis* and that it also shared a high sequence identity with GmMPK1 from soybean; AhMPK3 from peanut; CsTIPK from cucumber; NtWIPK from tobacco and LeMPK3 from tomato. The orthologs of CaMPK1 had been variously characterized in response to diverse forms of pathogen stresses and were also found to be responsive to phytohormones implicated in defense signaling pathways (Nakagami et al. 2005).

To study the relatedness and putative role of CaMPK1 more closely, it was studied in relation to MAPKs isolated and characterized from legumes, under various conditions. The sequence parameters of nine MAPKs from legumes, including those belonging to the model legumes like *Medicago* and *Glycine* were studied. The analysis revealed a great similarity in the arrangement and position of the conserved domains in CaMPK1, PsMPK3, AhMPK3, GmMPK1 and MsMMK4. The phylogenetic analysis of these MAPKs clearly clustered them into three distinct groups, as per the conventional classification (MAPK Group 2002), as Group A1, A2 and B2 MAPKs. CaMPK1 was clustered together with PsMPK3, AhMPK3, GmMPK1 and MsMMK4 as Group A1 MAPKs, further confirming the earlier observations.

The *in silico* analysis of the various sequence parameters of CaMPK1 confirmed that the isolated clone was a MAPK, the first to be isolated from chickpea. Further, it classified it as a Group A1 MAPK, based on its sequence identities. Although there were no previous reports of CaMPK1 or its homolog being isolated from chickpea, the genetic data available form its orthologs in model systems and other legumes indicated that it may be activated during the early intervals of pathogen recognition and wounding in plants.
Characterization of CaMPK1 in response to *Ascochyta* infection and defense signal modulators

The strong body of evidence suggesting the role of Group A1 members in innate defense against pathogen attack led us to investigate the role of CaMPK1 during *Ascochyta* infection. With this objective, the transcript accumulation of CaMPK1 was assayed in both resistant (FLIP84-92C) and susceptible (P-362) chickpea lines during *Ascochyta* infection. The results showed an early and positive accumulation of CaMPK1 transcripts in both the lines, during the disease cycle. Further, the expression of CaMPK1 was more pronounced in the resistant varieties as compared to the susceptible. These results also correlated to similar observations in its *Arabidopsis* ortholog AtMPK3, induced in response to bacterial flagellin and the wilt fungus, *Fusarium oxysporum* (Asai et al. 2002; Berrocal and Molina 2008). The CaMPK1 induction was biphasic which was attributed to the dual processes of PAMP recognition followed by the oxidative burst in response to the pathogen attack, also observed in similar plant-pathogen interactions (Reinold and Hahlbrock 1996; Berrocal and Molina 2008). These results confirmed the transcriptional activation of CaMPK1 in response to *Ascochyta* infection of chickpea. Thus the role of CaMPK1 as an early responsive defense-signaling gene, elicited in response to the detection of *Ascochyta* infection was experimentally confirmed.

Due to the presence of various transcriptional regulators, the transcriptional activity of genes does not always correlate to the activity of its corresponding protein. To investigate and verify the translational activity of CaMPK1 during *Ascochyta* blight, its recombinant protein was cloned and expressed as an 85 kDa fusion product, to be used for further studies. The recombinant protein was verified for its innate kinase activity and was found to auto-phosphorylate as well as phosphorylate its downstream substrates in kinase assays, as reported in most cloned plant MAP kinases (Veronese et. al. 2006). The preliminary assay of rCaMPK1 incubated *Ascochyta* infected protein samples from chickpea showed the active phosphorylation of rCaMPK1 during the different phases of *Ascochyta* infection. Further, the protein phosphorylation profile correlated with the transcript accumulation of CaMPK1, during *Ascochyta* infection. The results also indicated the activation of a MAPK cascade in the *Ascochyta* infected chickpea samples that was able to specifically activate CaMPK1, its downstream substrate.
To confirm the specific activation of the rCaMPK1 in by the MAPK cascade activated in response to *Ascochyta* infection of chickpea, an in gel assay was performed using rCaMPK1 as the substrate. The results showed the phosphorylation activity of rCaMPK1 by components of the MAPK cascade activated in response to *Ascochyta* infection. The differential phosphorylation of rCaMPK1 was indicative of the differential levels of the active upstream MAPK components in the various *Ascochyta* infected chickpea protein samples. Further, it also revealed the modulation of the protein activity of CaMPK1 by its upstream components, which was in correlation with the transcript accumulation of *CaMPK1* during chickpea-*Ascochyta* interactions. Thus the results confirm the activation of CaMPK1 and its upstream MAPK cascade components during the early events of chickpea-*Ascochyta* interactions.

The defense signaling pathways are modulated by different phytohormones that behave as local and systemic signals during defense responses (Koomneef and Pieterse 2008). To investigate the modulation of *CaMPK1* by these defense regulators, its transcript accumulation was checked in response to the exogenous application of these hormones. The SA treatment of chickpea plants led to the high accumulation of *CaMPK1* transcripts in a biphasic manner first from the 0.5h to 3h intervals and subsequently at the 12 and 24h intervals. Similar kinetics has been reported for several SA responsive genes, as SA is a potent signal promoting local oxidative burst upon pathogen detection (Berrocal and Molina, 2008). The first induction in this case corresponds to the SA accumulation whereas the second induction is triggered by the SA-mediated oxidative burst (Glazebrook, 2005; Berrocal and Molina 2008). JA treatment of chickpea failed to elicit responses in *CaMPK1* corroborating the observations in its ortholog *AhMPK3* (Raja et. al. 2009). Thereby it was concluded that *CaMPK1* was not activated in response to JA based signaling. *CaMPK1* was activated in the later intervals in response to ET. In the background of the available genetic data it was concluded that *CaMPK1* might contribute to ET-based defense signaling, during the plant pathogen interaction, in coordination with other regulators like SA. The mechanical wounding executed to mimic the pathogen induced wounding led to the early accumulation of *CaMPK1* transcripts. Wounding generated systemic peptide systemin is a potent promoter of early oxidative burst, in cultured tomato cells (Stennis et al. 1998). Thereby, *CaMPK1*
might lead to activation of oxidative burst in response to pathogen induced wounding during *Ascochyta*-chickpea interactions. To conclude, CaMPK1 was activated during the early responses of chickpea to *Ascochyta* infection, both at the transcript and the protein levels. The *CaMPK1* appears to be positively regulated by SA and wounding during the initial defense responses, whereas it may regulate downstream players in the disease cycle in coordination with SA and ET. Thus, the activation of CaMPK1 during the early events of *Ascochyta* infection and its modulation by the major defense signaling molecules was experimentally established.