Chapter 2: Review of literature
Plants being sessile organisms have developed sophisticated signalling machineries to adapt to the changing environment by regulating their cellular metabolism. A common mechanism plants use to translate the external stimuli into cellular responses are the activation of mitogen-activated protein kinase (MAPK) cascade. These protein kinase cascades are highly conserved in eukaryotes and consist of three subsequently acting protein kinases, a MAP kinase kinase kinase (MEKK), a MAP kinase kinase (MKK) and a MAPK (MPK) which are linked in various ways with upstream receptors and downstream targets.

2.1 MAPK cascade in general
Integration of the myriad cellular processes that enable eukaryotic organisms to grow and reproduce successfully requires the coordinated activity of an elaborate matrix of signal transduction proteins, within which the most prominent super-family consists of the protein kinases (PK). Within this super-family, the mitogen-activated protein kinases (MAPKs/MPKs) form a distinctive and highly conserved PK sub-family. Phosphorylation/dephosphorylation of proteins are among the most common post-translational modifications in all organisms. Protein phosphorylation carried out by protein kinases are typically organized and specific in various signalling cascades. It is estimated that about 30% of all proteins are phosphorylated in a eukaryotic cell, about 5% of the genomes of green plants (Viridiplantae) encode for protein kinases (The Arabidopsis genome initiative, 2000; International rice genome sequencing Project, 2005) and 10% of all plant kinases are involved in MAPK pathways. The MAPK cascade is a major cellular device by which eukaryotic cells adaptively respond to extracellular stimuli. These are conserved signalling modules found in all eukaryotic cells, including plants, fungi and animals. A MAPK cascade minimally consists of three kinases: a MAPKKK/MEKK (MAPK kinase), a MAPKK/MKK/MEK (MAPK kinase) and a MAPK, which phosphorylate and in turn activate, each other in a specific way. MAPKKKs are serine/threonine kinases phosphorylating two amino acids in the S/T-X_{3-5}-S/T motif of the MAPK activation loop. MAPKKs are dual specificity kinases that activate a MAPK through double phosphorylation of the T-X-Y motif in the activation loop. MAPKs are serine/threonine kinases able to phosphorylate a wide range of substrates, including other kinases and/or transcription factors. A fourth level of kinases, named MAPKKKKs (MAPKKK kinases), may act as adaptors linking upstream signalling steps to
the core MAPK cascades. Interactions between kinases within a MAPK cascade occur through docking sites present in the kinases and/or with the help of external scaffolding proteins. The typical events of a MAP kinase cascade initiate at the plasma membrane binding of a ligand to a receptor, which in turn leads to the activation of a G protein. The GTP-bound form of the G protein activates a MAP kinase kinase kinase (MEKK), which in turn phosphorylates and activates a MAP kinase kinase (MKK). The terminal component of the kinase module, the MAP kinase, is phosphorylated by MEK/MKK and subsequently translocates to the nucleus where it phosphorylates transcription factors, or targets cytosolic proteins such as other kinases or cytoskeletal-associated proteins (Calderini et al. 2001). A generalised scheme illustrating the different elements in MAP kinase signalling is shown in fig 2.1.

Fig. 2.1 Generalized mitogen-activated protein (MAP) kinase signal transduction cascade. Signals perceived by receptors are transduced through signalling intermediates to the first core kinase, MAP/ERK kinase. Activated MEK kinase phosphorylates and thus activates MEK on conserved serine (S) and threonine (T) residues. Active MEK phosphorylates and activates MAP kinase on conserved T and tyrosine (Y) residues. MEK kinase, MEK and MAP kinase may be held together in a signalosome complex by scaffold proteins. Activated kinases are de-activated by specific phosphatases. Active MAP kinase is released from the signalosome, dimerizes and activates by phosphorylation target proteins in the cytoplasm or nucleus. P, phosphorylated aminoacid. (Source: (Morris, 2001)
Moreover, MAPK modulators such as phosphatases are involved in the control of signal strength and the duration of signalling processes. MAPK phosphatases (MKPs) regulate the MAPK pathway either by time dependent control or by the shut-down of the pathway after signalling. The 80 MAPKKKs are heterogeneous group of protein kinases and divided into three main subgroups: the MEKK (MAPK/ERK kinase kinase)-like MAPKKKs, for which functional evidence is there that they act as MAPKKKs in planta, and the Raf-like and plant M KK related ZR1 interacting kinases (ZIK) like subgroups, for which the only functional evidence comes from non-plant systems. In addition to their kinase activity, plant MAPKKKs often contain long N or C terminal regions that might function in regulation or scaffolding to recruit MAPKKs and MAPKs, or in the integration of the input signals. Apart from identifying a MAPKK as a scaffold, no specific scaffolding protein was yet reported in plants (Champion et al. 2004).

2.2 Classification of MKK

MAPKs and MAPKKs constitute homogeneous kinase families, mechanistically behaving like their homologues in animals and yeast and divided into four subfamilies (A–D) based on sequence similarities (MAPK group, 2002). *Arabidopsis* possesses genes encoding 20 MAPKs and 10 MAPKKs as compared to 6 MAPKs and 6 MAPKKs in yeast and 10 MAPKs and 7 MAPKKs in human. The *Arabidopsis* genome was originally anotated as possessing 10 members of the MKK gene family (MAPK group, 2002), but closer examination of those sequences revealed AtMKK10 lacks a properly constructed activation loop target site, which raises the question of its biological functionality. Rice has 8 MKKs grouped into 4 subgroups. In addition to the thirteen poplar and rice MKK gene models possessing fully canonical motif structures (Jonak et al. 2002), an additional 6 MKK genes (three each in poplar and rice) are deficient in one or more motif elements. Phylogenetic analysis of the MKK gene families in the three species places the MKK genes in generally well-resolved clades that each contains members from both monocots and eudicots, with the exception of the MKK7-9 clade, of which no rice ortholog can be identified. A five-member sister group of MKK7-9 clade includes AtMKK10, a poplar ortholog (putative PtMKK10) and three paralogous rice sequences (putative OsMKK10-1, OsMKK10-2 and OsMKK10-3),
possesses an incomplete activation loop motif in which the 5’-S or T residue is either absent or located 3–5 residues upstream of the canonical position. Although these five genes share a common evolutionary history, the differences in the amino acid sequence of the MKK activation loop of PtMKK10 and that of the monocot ortholog OsMKK10 are fewer than the differences seen between the same region of PtMKK10 and AtMKK10, even though the two eudicot species are evolutionarily more closely related. Expression analysis suggests mooted functionality because no convincing evidence was found in the databases for transcription of AtMKK10, PtMKK10, PtMKK11-1, PtMKK11-2, OsMKK10-1 or OsMKK10-3. Interestingly, OsMKK10-2 is expressed in several tissues, and corresponding ESTs have been found for rice and maize. A less complex case involves AtMKK8, a gene whose encoded protein possesses all the canonical MKK motifs, but for which no evidence of expression was found in any of the three assay systems. Neither the poplar nor the rice genome sequences have a clear ortholog of AtMKK8, suggesting that ancestral gene duplication occurred after the divergence of the monocots and eudicots which could have yielded the precursors of the AtMKK7-8-9 and the PtMKK7-9-11 clades. Since that time, both PtMKK11-1 and PtMKK11-2, and AtMKK8 might have drifted toward a non-functional state, which would be consistent with the large evolutionary distances in the phylogenetic reconstruction. As a result, the functional Arabidopsis MKK gene family is likely to consist of just eight members, as does the poplar family. One of the more interesting MKK clades consists of the MKK3 sequences. These genes are unusual in their possession of a 30 extension encoding a NTF (‘nuclear transfer factor’) domain. Nuclear Transport Factor2 (NTF2) is a small protein that mediates the nuclear import of RAN-GDP and binds to both RAN-GDP and FxFG repeat–containing nucleoporins (Quimby et al. 2000). Although NTF proteins are found encoded in other eukaryotic genomes, including Arabidopsis, the combination in plants of a MAPKK and a NTF within a single gene product appears to be unique among eukaryotic taxa. No biological functions have yet been associated in plants with either MKK3, or the NTF domain, but the MKK3 genes are actively transcribed in all three species analyzed. Interestingly, the Chlamydomonas genome encodes a single MKK and this MKK belongs to the MKK3 structural class, including the 30-NTF domain, indicating that this chimeric arrangement has had a long and successful evolutionary history
in the lineage of photosynthetic eukaryotes. The combination of a MAPKK and an NTF2-like domain in plants appears to be unique among eukaryotic taxa.

2.3 Structural features of MKK
Mammalian mitogen-activated protein kinase (MAPK) cascades control various cellular events, ranging from cell growth to apoptosis, in response to external stimuli. MKK contains the 11 conserved catalytic subdomains and plant specific MAPKK sequence S/TXXXXXS/T motif between subdomain VII and VIII and it has a putative MAPK docking domain K/R-K/R-K/R-X_{1-6}-LX-L/V/I. The D-domain (Conserved docking domain) is highly conserved in animals, yeasts and plants (Bardwell et al. 1996). A conserved docking site, termed DVD, is found in the mammalian MAP kinase kinases (MAPKKs) belonging to the three major subfamilies, namely MEK1, MKK4/7, and MKK3/6. The DVD sites bind to their specific upstream MAP kinase kinase kinases (MAPKKKs), including MTK1 (MEKK4), ASK1, TAK1, TAO2, MEKK1, and Raf-1. DVD site is a stretch of about 20 amino acids immediately on the C-terminal side of the MAPKK catalytic domain. Mutations in the DVD site strongly inhibited MAPKKs from binding to, and being activated by, their specific MAPKKKs, both in-vitro and in-vivo. DVD site mutants could not be activated by various external stimuli in-vivo. Synthetic DVD oligopeptides inhibited specific MAPKK activation, both in-vitro and in-vivo, demonstrating the critical importance of the DVD docking in MAPK signalling as shown in Fig. 2.2. (Takekawa et al. 2005).

![Fig 2.2](image)

Fig 2.2 A schematic model for the DVD and CD docking interactions in mammalian MAP kinase cascades. Specific DVD docking interactions between MAPKKKs and MAPKKs, and CD docking interactions between MAPKKs and MAPK, ensure proper and efficient flows of signal through various MAPK cascades [Source: Takekawa et al. 2005].
2.4 History of MAP kinase cascades

MAP kinases first came to light when Sturgill & Ray (1986) identified a protein kinase from insulin-treated 3T3-L1 cell extracts that would phosphorylate microtubule associated protein-2 (MAP-2) on both serine and threonine residue. MAP-2 kinase was shown to be closely related to a set of previously identified proteins which are tyrosine phosphorylated in response to mitogens (agents which promote cell division) and was renamed p42 MAP kinase (Mitogen-Activated-Protein kinase) (Rossomando et al. 1987). It was later shown that the activity of the p42 MAP kinase protein was itself dependent on phosphorylation of both threonine and tyrosine (Anderson et al, 1990). Boulton et al. (1990) isolated and cloned an insulin activated protein kinase from chinese hamster ovary cells, named Extracellular signal Regulated Kinase 1 (ERK1). Several plant homologs of MEKs and MAPKs have been identified based on sequence similarity to the yeast and animal enzymes (Hirt, 1997; Mizoguchi et al. 1997). A plant MEK homolog was first identified in tobacco (Shibata et al. 1995), and in Arabidopsis, five MEK homologs have been identified: AtMEK1, AtMKK2, AtMKK3, AtMKK4, and MBP-AtMAP2Ka (Jouannic et al. 1999; Mizoguchi et al. 1997; Morris et al. 1997; Ichimura et al. 1998). Phylogenetic analysis indicates that these five MEK homologs belong to three subgroups.

Rice, one of the most important of all food crops worldwide, and a monocot cereal crop research model (Goff et al. 2002; Yu et al. 2002) has been the focus of studies aimed at defining the defense/stress responses (Agrawal et al. 2001; Agrawal et al. 2002). The completion of sequencing projects revealed 15 MAPK genes and 8 MAPKK genes in the Oryza sativa genome (Ichimura, 2002; Hamel et al. 2006). In Arabidopsis, six of the known MAPKKs have been found to be involved in diverse functions: AtMKK1 and AtMKK2 mediate abiotic stresses such as cold, salt and wounding (Teige et al. 2004; Hadiarto et al.: 2006) AtMKK4 and AtMKK5 are activated by fungal elicitors (Lee et al. 2004; Takemoto et al. 2005), AtMKK6 and AtMKK7 are involved in cytokinesis and development, respectively (Soyano et al. 2003; Melikant et al. 2004; Dai et al. 2006) and AtMKK9 with AtMPK6 is shown to play an important role in regulating leaf senescence (Zhou et al. 2009). In rice, however, only one (OsMKK6) of the eight known MAPKKs has been partially characterized (Wen et al. 2002), and to date there is no report of any plant MAPKK being induced by biotic stresses such as insects and fungal pathogens. Elevated MAPK activities, assayed using
myelin basic protein as a substrate, are observed when plant cells are stimulated by wounding (Usami et al. 1995; Seo et al., 1995; Bogre et al. 1997; Zhang and Klessig, 1998), pathogen elicitors (Suzuki and Shinshi, 1995; Ligterink et al. 1997; Stratmann and Ryan, 1997; Zhang and Klessig, 1997; Zhang et al. 1998; Romeis et al. 1999), or extracellular stresses (Jonak et al. 1996; Mizoguchi et al. 1996). MAPKs are also postulated to act in the signalling pathways for the hormones auxin, abscisic acid (ABA), and ethylene (Mizoguchi et al. 1994; Knetsch et al. 1996; Kieber et al. 1993; K.ovtun et al. 1998).

Ichimura et al. (1998) isolated three *Arabidopsis thaliana* cDNA clones (ATMKK3, ATMKK4 and ATMKK5) encoding protein kinases with extensive homology to the mitogen activated protein kinase kinases (MAPKKs) of various organisms in the catalytic domain. ATMKK3 showed high homology (85% identity) to NPK2, a tobacco MAPKK homologue. ATMKK4 and 5 are closely related to each other (84% identity). The recombinant ATMKK3 and ATMKK4 were expressed as a fusion protein with glutathione S-transferase (GST) in *Escherichia coli*. Affinity purified GST-ATMKK3 and GST-ATMKK4 proteins contained phosphorylation activity, which shows that both the ATMKK3 and ATMKK4 genes encode functional protein kinases. Northern blot analysis revealed that the ATMKK3 gene expressed in all the organs. The levels of ATMKK4 and 5 mRNAs were relatively higher in stems and leaves than in flowers and roots. They determined the map positions of the ATMKK3, 4 and 5 genes on *Arabidopsis* chromosomes by RFLP mapping using P1 genomic clones.

### 2.5 Abiotic stress

Abiotic stresses, such as drought, salinity, extreme temperatures, high light intensities, exposure to heavy metals are serious threats to agriculture, as they adversely affect the growth and productivity of crop plants. Abiotic stress is the primary cause of crop loss worldwide, reducing average yields for most of major crops by more than 50% (Boyer, 1982; Bray et al. 2000). Most abiotic stresses directly or indirectly lead to the production of free radicals and reactive oxygen species, creating oxidative stress (Oberschall et al. 2000). The burden of environmental stress on crop plants is likely to increase because of the climate changes associated with global warming. Furthermore, with extension of crop cultivation to environments which are not optimal for the growth of crop plants, development of stress tolerant plants is becoming increasingly important (Kathuria et al. 2007). Abiotic stress resulting from water deficit, high salinity or periods of drought are the major selective forces
in plant evolution (Inze and Montague, 1995) and commonly constitute serious threats to agriculture.

Salinity is one of the most common stresses encountered by plants in environment. The ability to sense salt stress is essential for plants to launch the processes underlying acclimation (Sangwan and Dhindsa 2002). Soil salinity is a complex phenotypic and physiological phenomenon in plants imposing ion imbalance or disequilibrium, hyperionic and hyperosmotic stress, thus disrupting the overall metabolic activities and causing plant demise. Worldwide, more than 80 million hectares of irrigated land (representing some 40% of the total irrigated land in the world) have already been damaged by salt (Xiong and Zhu 2001). Salt stress leads to severe inhibition of plant growth and development, membrane damages, ion imbalances due to Na\(^+\) and Cl\(^-\) accumulation, enhanced lipid peroxidation and increased production of reactive oxygen species like superoxide radicals, hydrogen peroxide and hydroxyl radicals, which are scavenged by both enzymatic and nonenzymatic mechanisms. An important consideration to maintain homeostasis during stress condition is also to accumulate low molecular weight, osmotically active compounds called osmolytes in the cytosol in order to lower the osmotic potential inside the cell, a process called osmotic adjustment (OA). Such compounds include simple or complex sugars, sugar alcohols, polyols, inositols, quaternary amino compounds like glycine-betaine, proline and higher polyamines (PAs), particularly the triamine spermidine (Spd\(^{3+}\)) and tetramine spermine (Spm\(^{4+}\)) (Hasegawa et al. 2000). They serve as osmoprotectants under water deficit conditions, maintain membrane structure, have antioxidant role and act as free-radical scavengers preventing lipid peroxidation or as regulators of K\(^+\) channels in stomata. The plants challenged by salt stress also synthesize the phytohormone abscisic acid (ABA) as an adaptive response to reduce transpiration via stomatal closure (Finkelstein et al. 2002; Seki et al. 2007).

Temperatures above the normal optimum are sensed as heat stress (HS) by all living organisms. HS disturbs cellular homeostasis and can lead to severe retardation in growth and development, and even death. As sessile organisms, plants are constantly exposed to changes in temperature and other abiotic factors. Worldwide, extensive agricultural losses are attributed to heat, often in combination with drought or other stresses (Mittler, 2006). The accumulation of heat shock proteins (HSPs) under the control of heat stress transcription
factors (HSFs) is assumed to play a central role in the heat stress response (HSR) and in acquired thermotolerance in plants and other organisms (Kotak et al. 2007). Cold stress, which includes chilling (<20°C) and/or freezing (<8°C) temperatures, adversely affects the growth and development of plants and significantly constrains the spatial distribution of plants and agricultural productivity. Cold stress prevents the expression of full genetic potential of plants owing to its direct inhibition of metabolic reactions and indirectly, through cold-induced osmotic (chilling-induced inhibition of water uptake and freezing-induced cellular dehydration), oxidative and other stresses. Cellular membranes are fluid structures, and cold temperatures can reduce their fluidity, causing increased rigidity. Plant cells can sense cold stress through low temperature induced changes in membrane fluidity, protein and nucleic acid conformation and/or metabolite concentration (a specific metabolite or redox status) (Chinnusamy et al. 2005).

Rice exhibits enormous genetic variability in its sensitivity to salt stress. The indica varieties Pokkali and Nonabokra, having higher endogenous ABA levels during osmotic shock, are classified as highly salt-tolerant ecotypes, while the majority of high-yielding cultivars like M-1-48, IR-29, IR-72, IR-36 and Taichung Native-1 are salt-sensitive (Moonset al. 1995). In addition, the scented or aromatic rice varieties are an important export commodity worldwide and command premium or higher prices and increasing demand in both domestic and international rice markets over non-scented varieties due to the recognition of their good qualities like pronounced or fragrant odour and perfumed aroma. Most of these quality aromatic rice genotypes are susceptible to biotic and abiotic stresses with consequent low-yield potential and hence cannot be traded at international level.

2.5.1 MKK and abiotic stress
To adapt to salt stress, plants have developed specific mechanisms to withstand salinity (Mishra et al. 2006; Qiu et al. 2004; Xiong et al. 2002; Zhang et al. 2004; Zhu, 2001), such as the synthesis of stress hormones like ABA that triggers secondary responses and activates the expression of specific sets of genes that help plants tolerate abiotic stresses (Zhang et al. 2004; Zhu 2001). The functional role of the MAPK cascade in NaCl or osmotic stress signalling has been established in yeast and human cells (Jonak et al. 2002; de Nadal et al. 2002), but progress in plants has been primarily in correlating the activation of the MAPK proteins with these stresses (Jonak et al. 2002). The most complete MAPK cascade
functioning in abiotic stresses consists of the MEKK1 activating M KK2 and MPK4/MPK6. In the mkk2 background, cold and salt activation of MPK4 and MPK6 are impaired and mkk2 mutant plants are hypersensitive to cold and salt stresses. Using the protoplast expression system, the authors showed that M KK2 activates MPK4 in response to cold and salt (Fig 2.3) (Teige et al. 2004).

**Fig. 2.3** Signalling events of MKK in salt and cold stress (Source: Teige et al. 2004).

AtMEK1 activity increased slightly after 60-120 min, when Arabidopsis seedlings were exposed to 300mM NaCl treatment. AtMEK1 has a substrate specificity preferring AtMPK4 to AtMPK3 (Matsuoka et al. 2002). The mkk9 mutant of Arabidopsis produced by T-DNA insertion is salt insensitive, it will germinate on media containing up to 150 mM of NaCl. Similarly mkk9 mutant is insensitive to mannitol in the germination medium. Induction of expression of the stress related genes RD29 and RD22 after salt treatment is faster in the mutant, and for RD22, the expression is prolonged. Enhanced stress related gene expression in mkk9 is hypothesized to be the reason for the enhanced ABA and salt tolerance, suggesting M KK9 acts as a negative regulator of the abiotic stress response (Alzwiy and Morris, 2007).

ABA, drought and salt stress induced gene expression of CAT1 catalase is mediated by AtMEK1 by triggering H2O2 signal production. Both CAT1 expression and AtMEK1 activity were activated by ABA, drought and salt stresses. The mekl mutant totally blocked stress induced H2O2 production. Overexpression of AtMEK1 significantly promoted stress induced CAT1 expression and also promoted H2O2 production. mekl was found to exhibit a hypersensitivity to drought and salt stresses and correspondingly AtMEK1 overexpressed plants were found to be more tolerant to drought and salt stresses (Xing et al. 2007).
To define the mechanisms of how different signals can activate a common signalling pathway, upstream activators of SIMK, a salt stress and pathogen induced alfalfa MAPK, were identified. They compared the properties of SIMKK, a MAPK kinase (MAPKK) that mediates the activation of SIMK by salt stress, with those of PRKK, a distantly related novel MAPKK. Although both SIMKK and PRKK show strong interaction with SIMK, SIMKK can activate SIMK without stimulation by upstream factors. In contrast, PRKK requires activation by an upstream activated MAPKK kinase. SIMKK mediates pathogen elicitor signalling and salt stress, but PRKK transmits only elicitor-induced MAPK activation. Of four tested MAPKs, PRKK activates three of them (SIMK, MMK3, and SAMK) upon elicitor treatment of cells. However, PRKK is unable to activate any MAPK upon salt stress. In contrast, SIMKK activates SIMK and MMK3 in response to elicitor, but it activates only SIMK upon salt stress (Cardinale et al. 2002).

Kiegrl et al. (2000) reported isolation and characterization of the alfalfa MAPK kinase SIMKK (SIMK kinase). SIMKK encodes an active protein kinase that interacts specifically with SIMK, but not with three other MAPKs, in the yeast two-hybrid system. Recombinant SIMKK specifically activates SIMK by phosphorylating both the threonine and tyrosine residues in the activation loop of SIMK. SIMKK contains a putative MAPK docking site at the N terminus that is conserved in mammalian MAPK kinases, transcription factors and phosphatases. Removal of the MAPK docking site of SIMKK partially compromises but does not completely abolish interaction with SIMK, suggesting that other domains of SIMKK are also involved in MAPK binding. In transient expression assays, SIMKK specifically activates SIMK but not two other MAPKs. Moreover, SIMKK enhances the salt-induced activation of SIMK. These data suggest that the salt-induced activation of SIMK is mediated by the dual-specificity protein kinase SIMKK.

Rice is much more sensitive to low temperature as a result of its tropical origin. Male sterility is the most severe consequence among the many chilling-induced agronomic damages in rice production. The developmental stages from pollen formation to fertilization are the most vulnerable to low temperature throughout the life cycle of rice plants (Nishiyama, 1984). Exposure of rice plants at the tetrad stage to a moderately low temperature (12°C) for 4 day resulted in male sterility in 80% of spikelets (Satake and Hayase, 1970; Nishiyama, 1984). Microscopic observation of developing rice anthers...
suggested that one possible reason for the male sterility after low temperature treatment was the failure of anther development. The observed abnormalities included the cessation of anther development, the arrest of pollen development, anthers remaining within the flowers after anthesis, and partial or no dehiscence (Satake, 1976). Cytological observation revealed a dilatation of tapetal layers in chilling-treated rice anthers (Nishiyama, 1976, 1984). The dilatation of tapetal layer was accompanied by a vigorous augmentation of cytoplasmic organelles such as mitochondria, proplastids, Golgi bodies, and endoplasmic reticula (Nishiyama, 1976, 1984). Chilling temperature treatment also affects the physiological status of anthers. Nonreducing sugar content was found to increase rapidly, whereas the acid phosphatase activity decreased in the moderately temperature treated rice anthers (Nishiyama, 1984).

OsMEKI transcript levels were induced in rice anthers by 12°C treatment for 48 h. Similar OsMEK1 induction was observed in shoots and roots of seedlings that were treated at 12°C for up to 24 h. No induction of OsMEK1 transcripts was observed in 4°C treated seedlings. In contrast, rice lip19, encoding a bZIP protein possibly involved in low temperature signal transduction, was not induced by 12°C treatment but was induced by 4°C treatment. Among the three MAP kinase homologs cloned, only OsMAP1 displayed similar 12°C specific induction pattern as OsMEK1. A yeast two-hybrid system revealed that OsMEK1 interacts with OsMAP1, but not with OsMAP2 and OsMAP3, suggesting that OsMEK1 and OsMAP1 probably function in the same signalling pathway. An in-gel assay of protein kinase activity revealed that a protein kinase (approximately 43 kD), which preferentially uses myelin basic protein as a substrate, was activated by 12°C treatment but not by 4°C treatment. These leads to conclude that at least two signaling pathways for low temperature stress exist in rice, and that a MAP kinase pathway with OsMEK1 and OsMAP1 components is possibly involved in the signaling for the higher range low-temperature stress (Wen et al. 2002).

2.6 MKK and cell division
In synchronized tobacco cell cultures, the p43Ntf6 MAP kinase is activated at a late stage in mitosis, around the anaphase/early telophase transition, and localizes in the middle of two microtubule arrays characteristic of the phragmoplast, a plant-specific structure involved in
laying down the new cell wall. The timing of kinase activation and its intracellular localization suggest that p43Ntf6 plays a role in cell plate formation during cell division. With the aim of identifying possible partners for p43Ntf6 two-hybrid screening of a tobacco BY-2 cell suspension cDNA library using the p43Ntf6 mitogen-activated protein (MAP) kinase as bait resulted in the isolation of a cDNA encoding a protein with features characteristic of a MAP kinase kinase (MEK), which has been called NtMEK1. Two-hybrid interaction analysis and pulldown experiments showed a physical interaction between NtMEK1 and the tobacco MAP kinases p43Ntf6 and p45Ntf4, but not p43Ntf3. In kinase assays NtMEK1 preferentially phosphorylated p43Ntf6. Functional studies in yeast showed that p43Ntf6 could complement the yeast MAP kinase mutant mpkl when co-expressed with NtMEK1, and that this complementation depended on the kinase activity of p43Ntf6. Expression analysis showed that the NtMEK1 and NTF6 genes are co-expressed both in plant tissues and following the induction of cell division in leaf pieces. These data suggest that NtMEK1 is an MEK for the p43Ntf6 MAP kinase (Calderini et al. 2001).

2.7 MKK and stomatal development

Stomata are specialized epidermal structures that regulate gas (CO₂ and O₂) and water vapour exchange between plants and their environment. In Arabidopsis thaliana, stomatal development is preceded by asymmetric cell divisions, and stomatal distribution follows the one-cell spacing rule, reflecting the coordination of cell fate specification. Stomatal development and patterning are regulated by both genetic and environmental signals. Arabidopsis MPK3 and MPK6, two environmentally responsive mitogen-activated protein kinases (MAPKs), and their upstream MAPK kinases, MKK4 and MKK5, are key regulators of stomatal development and patterning. Loss of function of MKK4/MKK5 or MPK3/MPK6 disrupts the coordinated cell fate specification of stomata versus pavement cells, resulting in the formation of clustered stomata. Conversely, activation of MKK4/MKK5-MPK3/MPK6 causes the suppression of asymmetric cell divisions and stomatal cell fate specification, resulting in a lack of stomatal differentiation and further established that the MKK4/MKK5-MPK3/MPK6 module is downstream of YODA, a MAPKKK. The establishment of a complete MAPK signalling cascade as a key regulator of stomatal development and patterning advances understanding of the regulatory mechanisms of intercellular signalling.
events that coordinate cell fate specification during stomatal development (Wang et al. 2007). The constitutive and inducible overexpression of MKK9 cause premature senescence in leaves and in whole Arabidopsis plants. The premature senescence phenotype is suppressed when MKK9 is overexpressed in the mpk6 null background. When either of MKK9 or MPK6 is knocked out, leaf senescence is delayed (Zhou et al. 2009).

2.8 MKK and hormonal signalling

Plants are sessile organisms lacking any possible means of avoiding environmental challenges, and thus have developed a network of signaling events leading to defensive responses by producing defensive compounds. Jasmonic acid (JA) or its methyl ester (MeJA) is a plant signalling compound involved in the regulation of many stress responses and development (Turner et al. 2002). It is induced by a wide range of biotic and abiotic stresses, such as wounding, ozone exposure, water deficit and pathogen attack (Creelman and Mullet 1997, Pieterse et al. 1998, Staswick et al. 1998, Overmyer et al. 2000, Rao et al. 2000, Turner et al. 2002, Farmer et al. 2003, Rojo et al. 2003). The JA biosynthetic pathway has been well studied, and much information about the type and subcellular localization of its enzymes is available (Mueller 1997, Berger 2002, Turner et al. 2002, Li et al. 2005). In contrast, information about the JA signalling pathway is limited. The few signalling components described have been mostly identified by mutant screens for plants displaying either a reduced sensitivity to JA or a constitutive or enhanced response to JA (Weber 2002). Recent studies have revealed that some mitogen-activated protein kinase (MAPK) cascades are involved in the wound/JA signalling pathway (Zhang and Klessig 1998, Seo et al. 1999, Ichimura et al. 2000, Petersen et al. 2000, Matsuoka et al. 2002).

In tobacco NtMPK4 was activated by wounding along with two other wound responsive tobacco MAPKs, WIPK and SIPK. NtMPK4 was activated by salicylic acid induced protein kinase kinase (SIPKK), which has been isolated as an SIPK interacting MAPK kinase. In NtMPK4 activity suppressed tobacco, wound induced expression of jasmonic acid (JA) responsive genes was inhibited. NtMPK4 silenced plants showed enhanced sensitivity to ozone. Inversely, transgenic tobacco plants, in which SIPKK or the constitutively active type SIPKKEE was overexpressed, exhibited greater responsiveness to wounding with enhanced resistance to ozone. NtMPK4 was expressed preferentially in
epidermis, and the enhanced sensitivity to ozone in NtMPK4 silenced plants was caused by an abnormal regulation of stomatal closure in an ABA independent manner. These results suggest that NtMPK4 is involved in JA signalling and in stomatal movement (Gomi et al. 2005).

The MKK3–MPK6 cascade was shown to play a role in jasmonate dependent negative regulation of ATMYC2/JASMONATE-INSENSITIVE1 (JIN1). ATMYC2/JIN1 is a major positive regulator of JA inducible gene expression and essential for JA dependent developmental processes in Arabidopsis thaliana. Takahashi et al. (2007) identified a mitogen activated protein kinase (MAPK) cascade, MAPK KINASE 3 (MKK3)–MAPK 6 (MPK6), which is activated by JA in Arabidopsis. They also showed that JA negatively controls ATMYC2/JIN1 expression, based on quantitative RT-PCR and genetic analysis using gain of function and loss of function mutants of the MKK3–MPK6 cascade. These results indicate that this kinase unit plays a key role in JA dependent negative regulation of ATMYC2/JIN1 expression. Both positive and negative regulation by JA may be used to fine-tune ATMYC2/JIN1 expression to control JA signalling. Moreover, JA regulated root growth inhibition is affected by mutations in the MKK3–MPK6 cascade, which indicates important roles in JA signalling.

Semidominant Arabidopsis activation tagged mutant, budl, in which the expression of the MKK7 gene is increased (Mou et al. 2002). The increased expression of MKK7 in budl or the repressed expression in MKK7 antisense transgenic plants causes deficiency or enhancement in auxin transport, indicated that MKK7 negatively regulates polar auxin transport (PAT) (Dai et al. 2006). budl mutant has an elevated level of SA, and exhibits constitutive PR gene expression and enhanced resistance to both Psm ES4326 and H. parasitica Noco2. MKK7 expression induced by pathogen infection in wild type plants and silencing of MKK7 by antisense RNA expression not only compromises basal resistance but also blocks the induction of SAR, demonstrating that MKK7 is a positive regulator required for both basal resistance and SAR. Ectopic expression of MKK7 in local tissues induces PR gene expression and resistance to the bacterial pathogen Psm E4326 in systemic tissues, indicating that MKK7 activation may be involved in generating the mobile signal for SAR (Zhang et al. 2007).
Ethylene (C₂H₄) was the first example of a gaseous signalling molecule in biological systems, discovered more than a century ago. As a major plant hormone, it controls essential physiological processes, including germination; root, shoot and flower development; stress, defence and glucose responses; fruit ripening; and senescence (Neljubov et al. 1901; Bleecker et al. 2000; Chen et al. 2005; Guo et al. 2004; Alonso et al. 2004). Extensive genetic analysis of ethylene signal transduction in *Arabidopsis* has established a linear pathway connecting five receptors 2–7 to a single negative regulator, CTR1 (Keiber et al. 1993), and two key downstream positive components, EIN2 (Alonso et al. 2003) and EIN3 (Chao et al. 1997). CTR1 encodes a putative Raf-like MAPKKK and interacts with the ethylene response 1 (ETR1) receptor, but its biochemical activity and molecular actions are unclear (Clark et al. 1998; Huang et al. 2003). EIN3 and its closely related EIN3-LIKE1 (EIL1) are plant-specific nuclear transcription factors that initiate downstream transcriptional cascades for ethylene responses (Solano et al. 1998; Alonso et al. 2003; Yanagisawa et al. 2003). The identification of ethylene receptor, CTR1, EIN2 and EIN3 orthologues in diverse plant species suggests the evolutionary conservation of ethylene signalling. However, it remains unknown how CTR1 functions as a MAPKKK to regulate downstream positive signalling components in the nucleus. EIN3 interacts with two F-box proteins (EBF1 and EBF2) and is degraded by the 26S proteasome (Potuschak et al. 2003; Guo et al. 2003; Gagne et al. 2004; Binder et al. 2007). Ubiquitin/proteasome dependent protein degradation mediated by specific F-box proteins of the conserved SCF (SKP1/Cullin/F-box protein) E3 ubiquitin ligase complexes has emerged as a universal mechanism in response to multiple plant hormones, including auxin, gibberellin, abscisic acid, jasmonate and ethylene (Dreher et al. 2007; Smalle et al. 2004). *Arabidopsis* MPK6 is also shown to phosphorylate and stabilize ethylene biosynthetic enzymes, 1-aminocyclopropane-1-carboxylic acid (ACC) synthase (ACS2/6), to promote ethylene synthesis (Liu et al. 2004). M KK9–MPK3/6 cascade positively modulates nuclear EIN3 stability. CTR1 acts as an unconventional MAPKKK, blocking M KK9–MPK3/MPK6 activation and simultaneously enhancing EIN3 degradation by distinct MPK phosphorylation. The results illuminate new molecular mechanisms for the control of transcription factor stability by intertwined MAPK cascades to achieve quantitative signalling specificity in eukaryotes (Fig. 2.4) (Yoo et al. 2008).
Xu et al. (2008) reported the identification of MKK9 as the upstream kinase of both MPK3 and MPK6 in planta. Activation of MKK9 leads to activation of the endogenous MPK3 and MPK6, and the subsequent up-regulation of multiple genes in ethylene and camalexin biosynthetic pathways and induces high levels of ethylene and camalexin accumulation. Mutation of either MPK3 or MPK6 compromises MKK9 induced ethylene and camalexin accumulation. In addition, activation of MKK9 enhances the sensitivity of transgenic seedling to salt stress. The results suggested that MKK9 plays an important role in ethylene and camalexin biosynthesis, and salt stress response in Arabidopsis.

Fig. 2.4 Summary of JA and ET biosynthesis and their signalling pathways in Arabidopsis. After stress perception plant cells activate protein phosphorylation cascades mediated by kinases. MAP kinase MPK6 phosphorylates target proteins, leading to cell responses. One of the targets is the enzyme responsible for ethylene production, ACS. ACS is stabilized by phosphorylation leading to enhanced ET production. ET receptors localized inside the cell at the endoplasmic membranes, bind ET and transmit the signal downstream to a specific kinase cascade that includes CTR1–MKK9–MPK3/MPK6, which conveys the signal to the nucleus to generate ET responses. At the same time MPK6 may modify other target(s) thereby leading to an enhancement of JA production (JA biosynthesis involves chloroplasts and peroxisomes). JA activates a specific MAPK pathway, which includes the MPK6 kinase. This signal is transferred to the nucleus and responses to JA are generated. Both pathways lead to activation of cell defence. (Source: Schweighofer and Meskiene, 2008).
2.9 MKK and biotic stress

Plant pathogen interactions involve very complex mechanisms ensuring survival in the competition arena. During evolution, higher plants developed an innate immune system to detect and rapidly respond to pathogen aggression (Jones and Dangl, 2006). After pathogen infection and recognition of PAMPs/MAMPs (pathogen/microbial associated molecular patterns) in the plant cell environment, multiple levels of defence are activated. PAMPs are small molecules usually derived from abundant pathogen structures such as structural proteins or cell wall components that are shared by a large range of pathogen varieties. Within minutes of pathogen derived PAMP recognition, the plant modifies enzymatic activities and gene expression patterns to synthesize a large set of antimicrobial reagents, including AOS (active oxygen species) and phytoalexins. In order to suppress the innate immune response of plants, pathogens inject a number of virulence factors into the host cell. In specific cases, however, plants learnt to recognize virulence factors, resulting in the so called ‘gene-for-gene resistance reactions’. Recognition of virulence factors occurs by plant NBS-LRR (nucleotide binding site–leucine rich repeat) receptors to switch on defence signalling cascades. Whereas PAMP recognition is not specific to a given pathogen variety, gene-for-gene interactions are based on the recognition of a given pathovar. Among others factors, resistance against different pathogen types generally involves a regulation of the balance between SA (salicylic acid) and ET/JA (ethylene/jasmonic acid) dependent defence mechanisms and thereby long term responses to pathogens (Glazebrook, 2005). SA is linked to resistance to biotrophic pathogens and is important to trigger the HR (hypersensitive response), a PCD (programmed cell death) to locally counteract pathogen attack and progression. ET and JA play a role in the control of PCD spreading (Overmyer et al. 2003) and regulate resistance against necrotrophic pathogens. All three hormones regulate distinct sets of pathogen-related genes and are involved in inducing SAR (systemic acquired resistance), a long-range process of priming pathogen resistance in unaffected tissues.

The best characterized plant MAMP receptor is the LRR receptor kinase FLS2 that perceives a conserved 22 amino acid peptide (flg22) from bacterial flagellin and activates MAPK cascade (MEKK1, MKK4/MKK5 and MPK3/MPK6) and WRKY transcription factors in Arabidopsis as shown in Fig 2.5 (Asai et al. 2002; Gomez-Gomez and Boller, 2000). MEKK1 is required for flg22 and/or reactive oxygen species (ROS)-induced MPK4
activation (Ichimura et al. 2006; Nakagami et al. 2006; Suarez-Rodriguez et al. 2007). MKK1 was also shown to be involved in flg22 induced activation of MPK4 (Meszaros et al. 2006).

![Diagram of innate immune signalling activated by bacterial PAMP flg22 in Arabidopsis. A putative repressor (R) could control WRKY22 and WRKY29 activity because their overexpression bypasses the requirement of elicitors. (Source: Asai et al. 2002)]

The mpk4 mutant plants exhibit a constitutive SAR phenotype, including elevated levels of SA, constitutive expression of PR genes and increased resistance to pathogens indicating MPK4 is a negative regulator of SAR (Petersen et al. 2000). SA and various pathogen-derived elicitors were shown to induce the tobacco mitogen-activated protein kinases (MAPKs), SA-induced protein kinase (SIPK) and wound-induced protein kinase (WIPK) (Zhang and Klessig, 1997). Silencing of NPK1 encoding MAPKKK, interferes with the function of the disease-resistance (R) genes N, Bs2 and Rx (Jin et al. 2003). Silencing of NTF6/ NRK1 (MAPK) or MEK1/NQK1 (MAPKK) attenuates N-mediated resistance to tobacco mosaic virus (Liu et al. 2003). In Nicotiana benthamiana NbMKK1–NbSIPK cascade was shown to control non-host resistance including HR cell death (Takahashi et al. 2007). Silencing of genes encoding two MAPKKs (LeMKK2 and LeMKK3) and two MAPKs (LeMPK3 and one similar to Ntf6) compromises Pto-mediated resistance (Ekengren
et al. 2003). Both LeMKK2 and LeMKK4 can phosphorylate LeMPK1, LeMPK2 and LeMPK3 in-vitro (Pedley and Martin, 2004).

MKK1 and MKK2 function upstream of MPK4. The double loss of function mutant (mkk1/mkk2) of MKK1 and MKK2 showed marked phenotypes in development and disease resistance similar to those of the single mkk1 and mpk4 mutants. mkk1 or mkk2 single mutants appear wild type and basal level of MPK4 activity were not impaired in them. By biochemical and molecular analysis, the kinases were shown to have a role in jasmonate and salicylate dependent defense responses, mediated in part via the MPK4 substrate MKS1. Transcriptome analysis revealed overlapping effects of MKK1 and MKK2 on global gene expression patterns demonstrating both redundant and unique functions for MKK1 and MKK2 (Qiu et al. 2008).

The endogenous Arabidopsis MAP kinase kinase MKK1 is activated in cells treated with flg22, phosphorylates MPK4 in-vitro, and activates MPK4 in-vivo in protoplasts. In mkk1 mutant plants, the activation by flg22 of MPK4 and two other flg22-induced MAPKs (MPK3 and MPK6) is impaired. In the mkk1 mutant, a battery of both flg22-induced and flg22-repressed genes show altered expression, indicating that MKK1 negatively regulates the activity of flagellin-responsive genes. Intriguingly, in contrast to the mpk4 mutant, mkk1 shows no morphological anomalies and is compromised in resistance to both virulent and avirulent Pseudomonas syringae strains. Thus, the MKK1 signalling pathway modulates the expression of genes responding to elicitors and plays an important role in pathogen defence. (Meszaros et al. 2006).

Gene coding for a blast (Magnaporthe grisea) and insect (Nilaparvata lugens) responsive putative MAPK kinase, OmMKK1 (Oryza minuta MAPKK1), identified in a library of O. minuta expressed sequence tags (ESTs). Two copies of OmMKK1 are present in the O. minuta genome encoding a predicted protein with molecular mass 39 kDa and pI of 6.2. Transcript patterns following imbibition of plant hormones such as methyl jasmonic acid (MeJA), ethephone, salicylic acid (SA) and abscisic acid (ABA), as well as exposure to methyl viologen (MV) revealed OmMKK1 expression is related to defense response signaling pathways. A comparative analysis of OmMKK1 and its O. sativa ortholog OsMKK1 showed that both were induced by stress-related hormones and biotic stresses, but
the kinetics of their responses differed despite their high amino acid sequence identity (96%) (You et al. 2007).

MKK2 is involved in both biotic and abiotic stress response. MKK2 is an upstream activator of MPK4 and MPK6 and plays a critical role in the cold and salt stress response in Arabidopsis, but does not mediate activation of these MAPKs by the elicitors flagellin and laminarin. However, transcriptome profiling of plants overexpressing wild type and constitutively active MKK2 revealed significant changes in the expression of a number of genes encoding proteins involved in transcriptional regulation, defense, signalling, and metabolism. Many of the affected genes overlapped with those found in cold and salt stress (Fowler and Thomashow 2002; Kreps et al. 2002; Seki et al. 2002), a significant number was linked to plant pathogen defense responses. MPK4 and MPK6 gets rapidly activated upon Pseudomonas syringae pv. tomato DC3000 infection, whereas plants expressing constitutively active MKK2 (MKK2-EE plants) have enhanced levels of MPK4 activation. MKK2-EE plants are more resistant to a virulent strain of P. syringae pv. tomato DC3000 and Erwinia carotovora subsp. carotovora SCC1, but hypersensitive to Alternaria brassicicola. In contrast, no differences in sensitivity were observed of mkk2 plants against E. carotovora subsp. carotovora SCC1 or A. brassicicola. Hormone analysis revealed that MKK2-EE plants were compromised in the production of jasmonic acid (JA) and salicylic acid (SA) upon infection by P. syringae pv. tomato DC3000 (Brader et al. 2007).

NtMEK2, a MAPK kinase, is upstream of salicylic acid-induced protein kinase (SIPK) and wounding induced protein kinase (WIPK), two tobacco MAPKs that are activated by various pathogens or pathogen-derived elicitors. Expression of a constitutively active mutant of NtMEK2 induces HR like cell death in tobacco, which is preceded by the activation of endogenous SIPK and WIPK. In addition, NtMEK2-SIPK, WIPK cascade control the expression of 3-hydroxy-3-methylglutaryl CoA reductase (HMGR) and L-phenylalanine ammonia lyase (PAL), two defense genes encoding key enzymes in the phytoalexin and salicylic acid biosynthesis pathways. The results demonstrated that a plant MAPK cascade controls multiple defense responses against pathogen invasion (Yang et al. 2001).

Transgenic ProMKK3:GUS lines (2 kb fragment upstream of the MKK3 translational start codon was fused to the β-glucuronidase (GUS) reporter gene and transgenic lines were generated) showed basal expression in vascular tissues and strongly induced by
*Pseudomonas syringae* pv tomato strain DC3000 (Pst DC3000) infection but not by abiotic stresses. The growth of virulent Pst DC3000 was increased in mkk3 knockout plants and decreased in MKK3 overexpressing plants. MKK3 overexpressed lines showed increased expression of several PR genes. By yeast two-hybrid analysis, coimmunoprecipitation, and protein kinase assays, MKK3 was revealed to be an upstream activator of the group C MAPKs MPK1, MPK2, MPK7, and MPK14. Flagellin derived flg22 peptide strongly activated MPK6 but resulted in poor activation of MPK7. By contrast, MPK6 and MPK7 were both activated by H2O2, but only MPK7 activation was enhanced by MKK3. In agreement with the notion that MKK3 regulates the expression of PR genes, ProPR1:GUS expression was strongly enhanced by coexpression of MKK3-MPK7. These results revealed that MKK3 pathway plays a role in pathogen defense and further underscore the importance and complexity of MAPK signalling in plant stress responses (Doczi et al. 2007).

Elicitins are small, secreted proteins produced by species of the plant pathogenic oomycete *Phytophthora*. They induce hypersensitive cell death in most *Nicotiana* species and in some cultivars of *Brassica rapa* and *Raphanus sativus*. Two true-breeding Fast Cycling *B. rapa* lines showed severe necrosis (line 7-R) or no visible response (line 18-NR) after treatment with elicitin. Microscopic examination revealed localized cell death in line 18-NR plants, and expression levels of various defense marker genes were compared in both lines. The result suggested that both “responsive” and “nonresponsive” plants responded to elicitin but differed in the extent of the cell death response. Expression of a constitutively active form of *Arabidopsis* MAP kinase kinase 4 (AtMEK4-DD) also induced rapid development of confluent cell death in line 7-R, line 18-NR and showed no visible cell death. Elicitin responsive *Nicotiana* species and *R. sativus* cultivars showed significantly stronger cell death responses following expression of AtMEK4DD compared with nonresponsive species/cultivars. Line 7-R also showed higher sensitivity to toxin-containing culture filtrates produced by *Alternaria brassicicola*, and toxin sensitivity co-segregated with elicitin responsiveness, suggesting that the downstream responses induced by elicitin and *Alternaria* toxin share factors that control the extent of cell death. Elicitin responsiveness was correlated with greater susceptibility to *A. brassicicola* (a necrotroph) in *B. rapa* but less susceptibility to *Phytophthora nicotianae* (a hemibiotroph) in *Nicotiana*, suggesting a more extensive cell
death response could cause opposite effects on the outcomes of biotrophic versus necrotrophic plant pathogen interactions (Takemoto et al. 2005).

The active mutants of AtMEK4 and AtMEK5, two closely related MAPKK under the control of a steroid inducible promoter induced transgene expression by the application of dexamethasone leads to HR like cell death, which is preceded by the activation of endogenous MAPKs and the generation of hydrogen peroxide. Both prolonged MAPK activation and reactive oxygen species generation have been implicated in the regulation of HR cell death induced by incompatible pathogens. The prolonged activation of the MAPK pathway in cells could disrupt the redox balance, which leads to the generation of reactive oxygen species and eventually HR cell death (Ren et al. 2002).

The MAP kinase kinase 7 (MKK7) positively regulates plant basal and systemic acquired resistance (SAR). The activation tagged bud1 mutant, in which the expression of MKK7 is increased, accumulates elevated levels of salicylic acid (SA), exhibits constitutive pathogenesis-related (PR) gene expression, and displays enhanced resistance to both *Pseudomonas syringae* pv. maculicola (Psm) ES4326 and *Hyaloperonospora parasitica* Noco2. Both PR gene expression and disease resistance of the bud1 plants depend on SA, and partially depend on NPR1. The constitutive defense response in bud1 plants is a result of the increased expression of MKK7, and requires the kinase activity of the MKK7 protein. The expression of the MKK7 gene in wild-type plants is induced by pathogen infection. Reducing mRNA levels of MKK7 by antisense RNA expression not only compromises basal resistance, but also blocks the induction of SAR. Intriguingly, ectopic expression of MKK7 in local tissues induces PR gene expression and resistance to Psm ES4326 in systemic tissues, indicating the activation of MKK7 is sufficient for generating the mobile signal of SAR (Zhang et al. 2007).

2.10 Cross-talk between plant MAP kinases

MAP kinases play a central role in transduction of different types of signals. Plant MAP kinase cascade are not linear pathways but complex networks, which are necessary for many fundamental physiological functions like stress signalling, hormonal responses, cell cycle regulation and defense mechanism. The complexity is enhanced when the activator of MAP kinase pathway is itself regulating diverse plant responses. This is best exemplified by
phytohormones, which mediate developmental programmes in plants as well as plant responses to a large number of extracellular signals. Usually, more than one hormone is involved in regulating a physiological event. It is therefore evident that plant MAP kinase pathways are involved at various levels in plant responses to hormonal and environmental stimuli. Thus the MAP kinases are not neatly delineated into separate parallel cascades, instead they show lot of overlap and cross-talk. Fig 2.6 depicts schematic representation of the likely cross-talk between different plant MAP kinase signalling networks.

It seems that different stimuli may activate different yet related patterns of MAP kinases. The complexity is enhanced by the findings that each component of this three tier module is a multigene member allowing for functional redundancy between members of the same family (Fig 2.6). It has been observed that some MAP kinases are involved in the regulation of different responses, for example, active ANP-like MAPKKKs play a dual role in cell cycle regulation (Nishihama et al. 1997) and transduction of the oxidative stress signal (Kovtun et al. 2000). Yet others may be activated by common extracellular signals but have different signaling pathways, for example, wounding, cold and salt stress induces AtMPK4 and AtMPK6 activity (Ichimura et al. 2000) but AtMPK4 is activated by AtMEKK1 during wounding and by MKK2 in cold and salt stress (Ichimura et al. 1998). The Arabidopsis ANP and tobacco NPK1 (Nakashima et al. 1998), class of MAPKKKs, which are mainly involved during plant cell division, are the best characterized in plants. The ANP1, initiates a phosphorylation cascade involving two stress MAPKs, AtMPK3 and AtMPK6, while the substrates for NPK1 are MAPKK, NQK1 and the MAPK, NRK1 (Takahashi et al. 2004). These MAPKKKs also transduce oxidative stress as well as repress auxin-related signalling in plant cells (Kovtun et al. 1998). Kovtun et al. (2000) reported the operational existence of a MAPK module in plants for the first time by demonstrating that H2O2 stress can regulate the target genes both positively and negatively (Fig. 2.6). The role of ANP/NPK family in auxin signaling was also studied (Gomez-Gomez and Boller 2000). It was shown that auxin and cytokinin together stimulate mitotic divisions in mature leaf tissues resulting in the NPK1 transcript accumulation, however auxin or cytokinin alone could not do so (Nakashima et al. 1998). This indicates a regulatory role for NPK1 in auxin and cytokinin-stimulated cell division, by inhibiting the expression of a subset of auxin-induced genes. Similarly constitutively active ANP1, ANP2, and ANP3 suppressed auxin-regulated
promoters in *Arabidopsis* protoplasts (Kovtun et al. 1998). The complexity of MAP kinase signaling module became apparent by the observations that transgenic NPK1 plants show enhanced tolerance to cold, heat, and salt stress without activating previously described drought, cold, and abscisic acid-signaling pathways (Kovtun et al. 1998). In planta studies using *Arabidopsis* T-DNA insertion mutants showed that ANP family acts as a positive regulator of cell division and growth and may act as a negative regulator of stress responses (Krysan et al. 2002). Moreover, virus-induced gene silencing of the MAPKKK, NPK1, in *Nicotiana benthamiana* plants interfered with the function of several disease resistance genes (Sharma et al. 2003). Since ANP/NPK proteins are found at high levels in meristems (Nishihama et al. 1997), these MAPKKKs might mediate a natural tolerance of meristems to diverse stresses (Machida et al. 1998). It is possible that the ANP/NPK like MAPKKKs will target additional MAPKs genes. The specificity for signal transmission in this case is probably maintained at the lower level of the MAP kinase module. AtMPK3 and AtMPK6 are the specific substrate for ANP kinases that are activated during cell division (Fig. 2.6) and other MAPKKKs like CTR1 cannot activate these MAPKs. It was also discovered that *Arabidopsis* AtMPK6 or tobacco SIPK and *Arabidopsis* AtMPK3 or tobacco WIPK are activated in response to different abiotic and biotic stresses (Droillard et al. 2002). Seo et al. (1999) observed that SIPK-specific kinase activity could be immunoprecipitated from wounded tobacco leaves, but not WIPK. Zhang and Klessig, (1998) presented evidence that SIPK was activated in response to salicylic acid, wounding and with biotic stresses including different fungal elicitors. Additionally, WIPK was activated, a few hours later to SIPK activation (Zhang and Klessig, 2000). Furthermore, it was shown that besides non-specific elicitors, race specific fungal elicitors also activate the WIPK and SIPK kinases. The upstream kinase, which activates SIPK and WIPK has been isolated (Yang et al. 2001) and this will enable the functional analysis of the MAPKs under both biotic and abiotic stresses.
Fig. 2.6 Diagramatic representation of cross talk between different plant MAP kinase signalling pathway. The scheme of general signal transduction pathway is shown. The homologs in Arabidopsis (At), Tobacco (Nt), Parsely (Pc), and tomato (Le) are shown. '?' indicate unidentified MAP kinase components. FLS2 is the putative receptor for flagellin peptide elicitor, Flg22. The '+' indicate induction while '-' indicate repression. (Source: Mishra et al. 2006)

Various studies support the findings that the complexity or redundancy of the pathway is evident across plant species. In alfalfa, M KK1 (SIPK homolog) and M KK4 or SAMK might be involved in wound-responsive MAP kinase signaling (Zhang and Klessig 1998) in addition to their association with signalling of many different forms of stress (Bogre et al. 1997). It has been shown recently that the SIPK and WIPK homologs from tomato, LeMPK1, LeMPK2 and LeMPK3, respectively, are induced in response to different oligosaccharide elicitors, systemin, mechanical, and wounding stress (Fig. 2.5) (Mayrose et al. 2004). Overall MAPK cascade are highly conserved and have emerged as universal signal transduction components that link receptors/sensors to cellular and nuclear responses.

2.11 With no lysine Kinases (WNK)

One of the Protein kinases WNK (with no lysine (K)) kinases are a subfamily of serine/threonine protein kinases related to the STE20/PAK-like family (Xu et al. 2002). A lysine residue in kinase subdomain II, which is essential for the coordination of ATP in the active centre and conserved among all other kinases, is missing in this subfamily (Xu et al.
2002 and Wilson et al. 2001). It was shown to be replaced by a lysine residue in subdomain I that is characteristic for members of the WNK family (Xu et al. 2000). WNK kinases have been found in numerous eukaryotes, but are not found in yeast and it has been proposed that they are restricted to multicellular organisms (Xu et al. 2002 and Verissimo et al. 2001). Rat WNK1 was first mammalian member of this protein kinase family cloned and characterized (Xu et al. 2000). Four WNK family members have been identified in humans with high sequence identity within their kinase domains (Verissimo et al. 2001). A significant upregulation of WNK1 and WNK4 mRNA expression was detected in kidneys of rats kept for 6 weeks on high salt diet (8% NaCl) and suggested physiological role of WNKs in maintaining body fluid homeostasis.

Mutation in WNK1 and WNK4 is known to cause pseudohypoaldosteronism type II, a form of familial hypertension resulting from activation of single gene (Wilson et al. 2001). Northern analysis indicated that WNK1 is widely expressed in different tissue, whereas WNK4 is widely expressed primarily in kidney. (Xu et al. 2000; Wilson et al. 2001 and Verissimo et al. 2001). WNK1 contains an autoinhibitory sequence just C-terminal to the catalytic domain and this autoinhibitory domain suppresses the activity of the kinase domain, which is active once this domain is removed. Activation of WNKs requires autophosphorylation of serine at 382 positions in the activation loop (Xu et al. 2002). Overexpression of WNK1 in HEK293 cells causes activation of ERK5 and the activation is blocked by MEK5 inhibitor U0126 and is dependent on MEKK2/3 and places WNK1 in the ERK5 MAPK pathway upstream of MEKK2/3. Gain of function mutation of WNKs is associated with salt sensitive hypertension in human patients.

The model plant Arabidopsis has been reported to have nine members of the WNK family (Nakamichi et al. 2002) but only one of them has so far been characterized. AtWNK1 phosphorylate the putative circadian clock component APRR3 in-vitro and might be involved in a signal transduction cascade regulating its biological activity (Kojima et al. 2002). A member of Arabidopsis WNK family (AtWNK8) interacts with subunit C of the vacuolar H⁺-ATPase (V-ATP-ase) via a short C-terminal domain. AtWNK8 is shown to autophosphorylate intermolecularaly and to phosphorylate Arabidopsis subunit C (AtVHA-C) at multiple sites as determined by MALDI-TOF MS analysis (Hermesdorf et al. 2006).
The 24 hour periodicity of circadian rhythms enables organisms to coordinate their activities with the external light dark cycles by anticipating the coming of dawn or dusk. Circadian rhythms in plants include movement of organs, such as leaves, petals and stomata opening (Barak et al. 2000). Plants have adapted their growth and development to use the diurnal cycling of light and dark. This is manifested at both physiological and the molecular level with expression of some genes occurring only at certain times of the day. A number of circadian-regulated genes have been identified through extensive analysis with DNA microarrays of *Arabidopsis* (Harmer et al. 2000; Schaffer et al. 2001). The day/night cycling of gene expression is called diurnal rhythm and is achieved primarily by two mechanisms: first by light called diurnal and second by a free running internal circadian clock (Dunlap, 1999). Eleven percent of the genes, encompassing genes expressed at both high and low levels, showed a diurnal expression pattern and 2% cycled with a circadian rhythm (Schaffer et al. 2001). APPR3 is a substrate of *Arabidopsis* WNK1, the gene for which also shows a rhythmic transcriptome that is coincident with the APPR3 rhythm and provided molecular link between the putative clock component, APPR3 and WNK1 which is implicated as a signal transducer. Nakamachi et al. (2002) found that transcript of *Arabidiopsis* WNK1 and three other members (WNK2, WNK4 and WNK6) are under the control of circadian rhythms. The *Arabidopsis* WNK gene family also regulates flowering time by modulating the photoperiod pathway (Wang et al. 2008).