Analysis of Signaling from an Unusual MAPKK (AtMKK3) in *Arabidopsis thaliana*

by

Gregory Raymond Lampard

B.Sc. University of Guelph, 1999
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ABSTRACT

Plants have developed numerous ways of adapting to a wide variety of environmental conditions. Mitogen-activated protein kinases (MAPKs) are a class of signaling molecules that are involved both in the detection of fluctuating environments and initiation of appropriate responses. MAPKs and their upstream activators, MAPK kinases (MAPKKs) are also involved in many additional physiological events, including the response to phytohormones, cell growth, cell death, differentiation and cell cycle control. It is thought that signal specificity and integration amongst MAPK signaling modules often occurs at the MAPKK level. MKK3 is a particularly interesting MAPKK because it is phylogenetically distinct from other plant MAPKKs, and uniquely contains both a canonical MAPKK S/T/Y dual-specificity protein kinase domain and a 'Nuclear Transport Factor 2' (NTF2) domain. In this thesis I demonstrate, using a range of reverse genetics approaches, that MKK3 appears to be involved in the response of *Arabidopsis thaliana* to specific environmental stresses (salt, osmotic and heat stresses) and phytohormones (auxin and ABA), and may also have functions in plant development. Through protein interaction studies and *in vitro* activity assays, I also identified a potential novel negative regulatory influence of MKK3 on MAPK signaling modules involving three MAPKs, MPK1, MPK2 and MPK7. This pattern is the first such report for plant MAPK signaling modules. My protein interaction studies also revealed a possible role for the NTF2 domain in mediating the pair-wise interactions between MKK3 and each of MPK1, MPK2 and MPK7. These results provide a platform that should facilitate future studies of specificity and cross-talk amongst MKK3-associated MAPK signaling modules in higher plants.

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2,4-D	2,4-dichlorophenoxyacetic acid
3AT	3-amino-1,2,4-triazole
6XHis	hexameric histidine tag
ABA	abscisic acid
ACC	1-aminocyclopropane-l-carboxylic acid
ACO	ACC oxidase
ACS	ACC synthase
ARF	auxin response factor
BR	brassinosteroid
CA	constitutively active
CaMV	Cauliflower Mosaic Virus
CARE	*cis*-acting regulatory element
CDPK	calcium dependent protein kinase
CFP	cyan fluorescent protein
CHX	cycloheximide
DCL	dicer-like
DNA	deoxyribonucleic acid
ET	ethylene
FOA	5-fluoroorotic acid
GA	gibberellic acid
GEO	gene expression omnibus
GFP	green fluorescent protein
GO	gene ontology
GUS	β-glucuronidase
HA	hemagglutinin
HR	hypersensitive response
IAA	indole-3-acetic acid
JA	jasmonic acid/jasmonate
LB	Luria-Bertani
MAPK	mitogen activated protein kinase
MAPKK	mitogen activated protein kinase kinase
MAPKKK	mitogen activated protein kinase kinase kinase
MAPKKKK	mitogen activated protein kinase kinase kinase kinase
MBP	myelin basic protein
MeJA	methyl jasmonate
miRNA	micro RNA
MPSS	massively parallel signature sequencing
mRNA	messenger RNA
MS	Murashige and Skoog
NAA	naphthalenacetic acid
NEB	native elution buffer
NPA	1-naphthylphthalamic acid

NPB	native purification buffer
NTF2	nuclear transport factor 2
NWB	native wash buffer
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PR	pathogenesis related
RNA	ribonucleic acid
RNAi	RNA interference
RT	reverse transcription
SA	salicylic acid/salicylate
SAGE	serial analysis of gene expression
SAR	systemic acquired resistance
SAUR	small auxin up-regulated
SC	synthetic complete
SNP	sodium nitroprusside
TAIR	The Arabidopsis Information Resource
TAP	tandem affinity purification
TF	transcription factor
VIGS	virus-induced gene silencing
WT	wild-type
X-gluc	5-bromo-4-chloro-3-ondoyl-glucuronide
yeast two-hybrid	yeast two hybrid
YFP	yellow fluorescent protein
YTA	yeast tryptone ampicillin

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CHAPTER 1. GENERAL INTRODUCTION

INTRODUCTION

Since plants are sessile organisms, they have evolved mechanisms for tolerating a wide range of environmental conditions and defending themselves from herbivores and pathogens. This requires the ability to detect changes in environmental conditions, interpret the extent and direction of that change and induce an appropriate physiological response. Many integrated signaling networks are involved in these processes, including protein phosphorylation and modification pathways, ion fluxes, lipid signaling networks, altered redox responses and micro RNA synthesis (Droillard et al., 2000; Droillard et al., 2002; Jonak et al., 2002; Zhu, 2002; Chinnusamy et al., 2004; Rizhsky et al., 2004; Takahashi et al., 2004; West et al., 2004; Larkindale et al., 2005; Mahalingam et al., 2005; Nakagami et al., 2005; Zhao et al., 2005). These signaling networks must function cooperatively to control changes in gene expression patterns and altered protein, hormone and metabolite levels that ultimately evoke a particular cellular response. One class of signaling networks that has been extensively studied with respect to plant stresses comprises protein phosphorylation networks involving multiple types of protein kinases, including mitogen-activated protein kinases (MAPKs; Jonak et al., 2002; Nakagami et al., 2005).

Consistent with the importance of detecting and interpreting external stimuli, the Arabidopsis genome has been found to encode a large family of receptor-like kinases (>400) that could function to both sense environmental changes and activate downstream amplification and integration systems, including protein kinase/phosphatase networks. Translation of the output from these signal networks is thought to often involve post-translational modification of

transcription factors that then directly or indirectly modify the expression of target genes. The Arabidopsis genome thus also encodes a large number (~1500) of transcription factors, belonging to at least 11 different classes (Riechmann and Ratcliffe, 2000), and some of these proteins have been demonstrated to be downstream effectors of MAPK signaling pathways in plants (Liu and Zhang, 2004; Feilner et al., 2005; Yap et al., 2005).

One of the major responses to environmental stresses is stimulus-specific alteration of phytohormone metabolism. Several different phytohormones are known to play roles in modulating stress responses, including jasmonic acid, auxin, abscisic acid, ethylene and salicylate (Cheong et al., 2002; Himmelbach et al., 2003; Mahalingam et al., 2003; Anderson et al., 2004; Navarro-Avino and Bennett, 2005). Apart from roles in stress response, each of these hormones also functions in other aspects of plant biology, particularly growth and development, which makes them powerful agents for integrating short-term stress responses with longer-term development-based adaptive change (Himmelbach et al., 2003; Chen et al., 2005; Lorenzo and Solano, 2005; Woodward and Bartel, 2005; Mur et al., 2006). The developmental processes controlled by phytohormones involve the activation of signaling networks similar to those utilized in response to stress, including changes in ion status, lipid modifications and transcription of regulatory micro RNAs (Finkelstein and Gibson, 2002; Casimiro et al., 2003; Jenik and Barton, 2005; Schmitz and Theres, 2005; Kepinski, 2006). Furthermore, there is increasing evidence that both short- and long-term changes in phytohormone signaling and sensing are also linked to protein kinase signal transduction, particularly to MAPK signaling (Ahlfors et al., 2004; Ludwig et al., 2005; Nakagami et al., 2005). In this thesis, I have explored some of these connections, with a focus on the interplay between MAPK and phytohormone signaling networks in the context of the plant stress response.

MITOGEN-ACTIVATED PROTEIN KINASE SIGNALING

Mitogen-activated protein kinases (MAPKs) comprise a family of protein kinases found in all eukaryotic organisms (Widmann et al., 1999; Nakagami et al., 2005; Hamel et al., 2006). Collectively, these kinases are known to function in a plethora of circumstances including, but not limited to stress responses, hormone signaling, cell growth, death, differentiation and cell cycle control (Ichimura et al., 2002; Tanoue and Nishida, 2003; Pedley and Martin, 2005; Hamel et al., 2006). Along the trajectory from input signal to cellular response, MAPK signaling modules are situated in the intermediary section. An upstream activating factor triggers the activation of a MAPK signaling module through the phosphorylation of a MAPK kinase kinase (MAPKKK), which phosphorylates and activates a MAPK kinase (MAPKK), that in turn phosphorylates and activates a MAPK. The activated MAPK subsequently phosphorylates the downstream target(s) of the signaling module, which contributes to the elicited response (Widmann et al., 1999). Examples of demonstrated MAPK substrates include transcription factors, cytoskeletal proteins, protein phosphatases, metabolic enzymes and other protein kinases (Liu and Zhang, 2004; Feilner et al., 2005; Katou et al., 2005; Yap et al., 2005).

On the input side, the activation of MAPKKKs occurs via phosphorylation of one or more serine or threonine residues, either by an upstream activating kinase, or by auto-phosphorylation following displacement of an N-terminal auto-inhibitory domain (Widmann et al., 1999; Huang et al., 2003; Soyano et al., 2003). Studies in other eukaryotic systems have revealed that MAPKKKs can be activated following interactions with several types of proteins including G-proteins (Luttrell and Luttrell, 2003), protein kinase C (Garcia-Rodriguez et al., 2005), small GTPases (Minden et al., 1995), receptor kinases (Irie et al., 1994) and the MAPKKK kinases (MAPKKKK; Elion, 2000). In plants, relatively little is known about the upstream MAPKKK-

activating elements. Interaction between the tobacco kinesins, NACK1 and NACK2, and the MAPKKK, NtMPK1, has been shown to prevent auto-inhibition of NtNPK1, resulting in its activation (Soyano et al., 2003). Although other elements potentially acting upstream of MAPKKKs have been identified in plants on the basis of sequence homology to other organisms (Nakagami et al., 2005; Pedley and Martin, 2005), direct activation of specific plant MAPKKKs by these elements has yet to be reported.

Catalytic activation of a MAPKK by upstream MAPKKKs is achieved through the dual phosphorylation of serine or threonine residues in the S/TXXXXXS/T plant MAPKK signature motif (Ichimura et al., 1998). This motif differs slightly from the S/TXXXS/T signature motif found in other eukaryotic MAPKKs, but in all instances phosphorylation of both residues is required to obtain any detectable activity (Seger et al., 1992). Although activation of MAPKKs *in vivo* can be catalyzed by upstream MAPKKKs, MAPKKs also display auto-activation, at least *in vitro* (Ichimura et al., 1998). Apart from the consensus phosphorylation site, plant MAPKKs generally contain a basic, N-terminal MAPK docking motif [K/R][K/R][K/R]$X_{(1-5)}$[L/I]X[L/I] that is similar to the MAPK docking motif found in MAPKKs of other organisms (Ichimura et al., 2002). This site has been shown to be important for efficient interaction between MAPKKs and their cognate MAPK targets (Kiegerl et al., 2000).

Further downstream in the cascade, activation of a MAPK requires dual phosphorylation of a threonine and tyrosine residue in the MAPK signature motif, -TXY-, which is located in the catalytic domain (Widmann et al., 1999). While the precise order of phosphorylation of these residues does not appear to be important in controlling activation, phosphorylation of both is essential for catalytic activity (Widmann et al., 1999). Kinetic studies have illustrated that both phospho-transfer reactions required for MAPK activation can be catalyzed by the same upstream

dual-specificity MAPKK. However, they occur in a two-step process in which the MAPK temporarily leaves the active site of the MAPKK prior to phosphorylation of the second amino acid residue (Ferrell Jr. and Bhatt, 1997). As with MAPKKs, MAPKs can auto-activate *in vitro* in the presence of ATP but this property varies between MAPKs and the biological significance of their auto-activation is not known (Huang et al., 2000).

Signaling through MAPKs can be stopped by dephosphorylation of either of the phospho-threonine or phospho-tyrosine residues in the -TXY- motif, reactions that can be catalyzed by a range of protein phosphatases, including PP2Cs, PPA2s, protein tyrosine phosphatases and a unique class of dual-specificity, MAPK-phosphatases (Widmann et al., 1999).

Unlike the genomes of mammals, plant genomes encode only ERK-type MAPKs (Ichimura et al., 2002). Nonetheless, MAPK signaling may be involved in more cellular processes in plants than in other organisms since more members of each component of the MAPK signaling module are encoded in the Arabidopsis genome than in metazoan lineages. Approximately 60 Arabidopsis MAPKKKs, 10 MAPKKs and 20 MAPKs (Ichimura et al., 2002) have been identified, whereas the yeast and human genomes encode 4 and 14 MAPKKKs, 4 and 7 MAPKKs and 6 and 13 MAPKs, respectively (Widmann et al., 1999; Meskiene and Hirt, 2000; Samaj et al., 2004). Examination of other plant genomes, however, suggests that the overall scale of MAPK gene families is not exceptionally large in plants, although extensive gene duplication has amplified some clades (Hamel et al., 2006). The generally large ratio of MAPKKKs to MAPKKs suggests a crucial role for MAPKKs in the integration of incoming signals passing through MAPK modules, while the ratio of MAPKKs to MAPKs similarly implies that at least some Arabidopsis MAPKKs must be capable of phosphorylating multiple downstream MAPKs. In Arabidopsis, for example, both AtMKK4 and AtMKK5 appear capable

of activating MPK3 and MPK6 both *in vitro* and *in vivo* (Asai et al., 2002). However, while the activation patterns of MPK3 and MPK6 overlap, stimulus-specific activation of each kinase is also known to occur (Droillard et al., 2000; Kumar and Klessig, 2000; Samuel et al., 2000; Droillard et al., 2002; Liu et al., 2003; Zhou et al., 2004; Miles et al., 2005). Recent work in yeast, mammals, *C. elegans* and Drosophila has revealed that signaling specificity results, in part, from the formation of multi-protein complexes involving both kinases and one or more scaffolding proteins (Whitmarsh and Davis, 1998; Tanoue and Nishida, 2003). Scaffolding proteins may be non-catalytic proteins but components of the signaling machinery may also function as a scaffolding protein, as illustrated by Pbs2 in *S. cerevisiae* (Tanoue and Nishida, 2003; Saito and Tatebayashi, 2004). Pbs2 acts both as a MAPKK and as a scaffold protein promoting the assembly of the complete MAPK module (Tanoue and Nishida, 2003; Saito and Tatebayashi, 2004). To date, only a single scaffolding protein has been reported (but not published) in plants, which emphasizes the dearth of information concerning signaling specificity and cross-talk mechanisms in plants.

The apparent importance of MAPKKs as input integrators and as mediators of cross-talk between signaling modules makes this gene family a particularly interesting research target. Based on phylogenetic analysis, plant MAPKKs can be placed into four distinct groups, A-D, and the Arabidopsis genome encodes representatives of each class (Ichimura et al., 2002; Hamel et al., 2006). Arabidopsis group A MAPKKs include MKK1, MKK2 and MKK6, for each of which some functional information has been reported. MKK1 and MKK2 appear to be involved in both abiotic and biotic stress responses and both MAPKKs are able to activate MPK4 (Teige et al., 2004). On the other hand, MKK2, but not MKK1, can also activate MPK6 (Teige et al.,

2004). In addition to these stress-response roles for Group A MAPKKs, MKK6 is involved in cell division, with a specific association with cytokinesis (Soyano et al., 2003).

Group B MAPKKs are characterized by an extended C-terminal region containing a nuclear transport factor 2 (NTF2) domain. Group B is a single member clade in rice, poplar and Arabidopsis, where AtMKK3 is the Arabidopsis orthologue. To date, no functional data exists for Group B MAPKKs.

Group C MAPKKs include the two most extensively studied MAPKKs, MKK4 and MKK5. These two kinases appear to be at least partially redundant, since both can activate MPK3 and MPK6. The expression patterns of the corresponding genes also do not suggest discrete functions for these kinases since the patterns are largely overlapping. Both *MKK4* and *MKK5* are up-regulated in response to pathogen attack, ethylene and ozone (Genevestigator). The only marked difference between their expression profiles occurs during plant development. Genevestigator analysis shows that *MKK4* expression increases steadily throughout development, whereas *MKK5* expression remains relatively stable. Nonetheless, more detailed analyses of the biological functions of these kinases is needed before we can conclude full functional redundancy.

Arabidopsis representatives of Group D MAPKKs include MKK7, MKK8, MKK9 and MKK10, and it appears that at least two of these MAPKKs are involved in hormone signaling. MKK9 activation induces increased ethylene biosynthesis (Cluis, 2005) and MKK7 was recently reported to influence polar auxin transport (Dai et al., 2006).

Similar to MAPKKs, plant MAPKs can be placed into four phylogenetic categories (Ichimura et al., 2002; Hamel et al., 2006). The smallest group in Arabidopsis is Group A, which contains three members, MPK3, MPK6 and MPK10. MPK3 and MPK6, which are the most well-

characterized of the Arabidopsis MAPKs, are involved in many processes, including hormone responses and susceptibility to both abiotic and biotic stresses (Droillard et al., 2000; Kumar and Klessig, 2000; Samuel et al., 2000; Droillard et al., 2002; Liu et al., 2003; Zhou et al., 2004; Miles et al., 2005). Arabidopsis group B MAPKs include MPK4, MPK5, MPK11, MPK12 and MPK13. As with group A MAPKKs, group B MAPKs are involved in stress responses, both abiotic and biotic (MPK4), and in cytokinesis (MPK13).

Little information is available concerning the functions of the group C MAPKs, MPK1, MPK2, MPK7 and MPK14, but it has been suggested that MPK1 and MPK2 are involved in mediating auxin responses (Mizoguchi et al., 1994). In addition, microarray profiling experiments suggest that *MPK7* expression follows a circadian rhythm and that its promoter is responsive to ABA exposure and osmotic stress (Genevestigator).

Group D MAPKs are structurally unique in that they contain a -TDY- signature motif in their activation loop instead of the -TEY- motif seen in all other plant MAPKs, and they also possess extended C-termini (Ichimura et al., 2002). Although this is the largest group in Arabidopsis, including MPK8, MPK9, MPK15, MPK16, MPK17, MPK18, MPK19 and MPK20, no functional analyses have been reported. Analysis of the rice group D MPK, BWMK1, has shown that, as with TEY MAPKs, it is involved in responses to biotic stress (Cheong et al., 2003).

The majority of papers published on plant MAPK signaling have focused on the activation of a specific MAPK during the plant's response to a particular stimulus, or on genetic definition of its participation in that response. This narrow focus is reflected in our relative lack of functional knowledge of complete MAPK signaling modules; i.e. those extending from a receptor or sensor through to one or more downstream effectors. Only a single pathway has been identified to this

extent in plants. Recognition of the bacterial elicitor flagellin 22 (flg22) by the Arabidopsis FLS2 receptor results in the activation of a MAPK module consisting of MEKK1, MKK4/5 and MPK3/6 (Asai et al., 2002). Signaling through this pathway eventually results in increased activity of the WRKY22 and WRKY29 transcription factors, which are known to be involved in the plant's response to various pathogens (Asai et al., 2002). Aside from this, only the previously described MAPK module required for cytokinesis has been characterized from the MAPKKK to the MAPK level, although neither the ultimate inducer of the module nor the downstream targets of the activated MAPK are known.

As a result of recent efforts directed at characterizing the events occurring downstream of MAPK activation, several MAPK substrates have now been identified (Liu and Zhang, 2004; Feilner et al., 2005; Katou et al., 2005; Yap et al., 2005). The most well-characterized plant MAPK substrates include several protein classes involved in pathogen, stress and hormone responses. *MKS1* encodes a protein of unknown function but is involved in pathogen resistance (Andreasson et al., 2005). ACS6, is an ethylene biosynthetic enzyme and over-production of an active form of this protein confers constitutive ethylene production (Liu and Zhang, 2004). NtWIF, and NtWRKY1 encode ARF and WRKY transcription factors respectively, that appear to be involved in the hypersensitive response (Menke et al., 2005; Yap et al., 2005). Several additional substrates of MPK3 and MPK6 were recently identified using protein microarrays spotted with recombinant proteins (Feilner et al., 2005). While several additional classes of proteins including transcription related proteins, transporters and histones were found to be MAPK substrates, the biological significance of this has not yet been explored. Nonetheless, it is clear that characterization of MAPK substrates will help identify the multitude of biological processes affected by MAPK activity.

PHYTOHORMONE SIGNALING

A common element in many MAPK modules appears to be a link to phytohormone signaling. Plant hormones influence a host of functions in the plant, where they often play roles in both developmental processes and stress responses. This cross-over is not surprising, given that environmental conditions strongly influence developmental programs. For example, abscisic acid (ABA) is required for germination, and can also suppress lateral root development (Finkelstein and Gibson, 2002; Himmelbach et al., 2003). Thus, plants growing in saline environments show diminished lateral root growth, a response that is coupled with increased ABA production in the plant. In the following sections, I briefly review our current knowledge of phytohormone signaling in plants, with a focus on the association of each hormone with stress responses.

Auxin

Auxin (indole-3-acetic acid; IAA), was first discovered in the 1920's, and is one of the most widely studied of all plant hormones (as cited by Benjamins et al., 2005). In the years since this discovery, biologists have confirmed that auxin is found at fluctuating concentrations in all tissues and that this variation in local concentration is an essential feature of auxin biology (Ljung et al., 2005). As its ubiquitous distribution implies, auxin affects most parts of the plant, but it plays particularly critical roles in root development, cell division, floral and seed development and apical dominance (Casimiro et al., 2003; Jenik and Barton, 2005; Leyser, 2005; Woodward and Bartel, 2005; Aloni et al., 2006). The primary endogenous sources of auxin have been thought to be the meristematic regions of the shoot apex (Ljung et al., 2005). From there, auxin is transported by passive and active transport mechanisms to the root tip, where it is then transported through the root cortex into the root epidermal tissues. This establishes a basipetal

auxin concentration gradient that is essential for proper cell elongation, gravitropic responses and lateral root formation (Blakeslee et al., 2005). However, it has recently been established that auxin biosynthesis is not restricted to shoot apices, but can also occur within all portions of the root, with the major source being the meristem of primary roots followed by meristematic tissue of lateral roots greater than four days old (Bhalerao et al., 2002). This information has yet to be integrated into older models of auxin action in the literature.

The current consensus is that auxin can be transported in a non-polar fashion along vascular strands via diffusion through the phloem, but that a major alternate form of auxin transport, namely auxin polar transport (reviewed extensively in Blakeslee et al., 2005) is also used. Polar transport is dependent on two factors, differential ionization of IAA in different cellular compartments, and asymmetric distribution of auxin efflux carriers. The pKa of IAA is 4.7, which means that IAA exists in both the protonated and unprotonated form at extracellular pH values (pH~5), but almost exclusively in its non-protonated form at cytosolic pH (pH~7). Protonated IAA is uncharged, and can therefore diffuse freely from the apoplast into the cytosol where, at neutral pH, it becomes deprotonated (charged) and thus unable to re-cross the plasmalemma by simple diffusion (Blakeslee et al., 2005). In order to leave the cell, auxin must be actively transported out by membrane-localized auxin efflux carrier proteins. However, these are not uniformly distributed over the cell surface, but rather are concentrated at the bottom (basipetal) end of the cell (Blilou et al., 2005). Once it has been transported out into the apoplast, IAA again becomes protonated and this cycle of inward diffusion followed by active transport to the apoplast can be repeated. The asymmetric distribution of the efflux carriers in the plasmalemma of xylem parenchyma cells creates a net movement of auxin in a basipetal direction (Blakeslee et al., 2005). Recently it was shown that, in addition to diffusive uptake of

auxin, auxin influx carriers also exist (Blakeslee et al., 2005; Santelia et al., 2005). These are thought to function in ensuring adequate rates of uptake of auxin by the cell, and possibly also in helping reduce "lateral" uptake of auxin by neighboring cells (Blakeslee et al., 2005). A role for MAPK signaling in this process was recently uncovered in which MKK7 appears to be a negative regulator of polar auxin transport (Dai et al., 2006).

Recent advances in microarray technology have allowed large-scale study of auxin-induced changes in gene expression, and these data have resulted in major additions to the repertoire of known auxin-induced genes. These include the small auxin up-regulated genes (SAURs) of unknown function, whose mRNA and encoded proteins both appear to be short-lived, and the GH3-related genes which encode proteins known to be involved in the formation of IAA conjugates (Raghavan et al., 2006). The GH3-related gene products induced by exogenous auxin have been suggested to be involved in dampening the auxin response (Raghavan et al., 2006). A third family of genes up-regulated by auxin include the Aux/IAA genes (Raghavan et al., 2006). These encode proteins that repress the transcription of several auxin-responsive genes by forming heterodimers with the auxin response factor (ARF) transcription factors and thereby stopping the latter from activating the expression of auxin-induced genes (Reed, 2001; Dharmasiri et al., 2005). Induction of transcription of the Aux/IAA genes by auxin application might therefore be predicted to have a negative impact on auxin-induced gene expression. However, the situation is somewhat more complicated than this. The SCF^{TIR1} protein was recently identified as an auxin binding protein (so-called auxin receptor) that also displays ubiquitin ligase activity (Dharmasiri et al., 2005). Upon binding auxin, Aux/IAA proteins are displaced from their cognate ARFs, rapidly ubiquitinated and degraded via the 26S proteasome, of which SCF^{TIR1} forms a part. This displacement and destruction of the Aux/IAA proteins frees

the ARFs to bind to the promoters of other auxin-induced genes and activate their transcription (Dharmasiri et al., 2005). Therefore, it appears that increased transcription of Aux/IAA genes following auxin application is actually part of a feedback mechanism that functions to restore the cell to homeostatic conditions.

It is becoming increasingly evident that biological responses are seldom controlled by a single plant hormone in isolation. Instead, fluxes in the levels of a given hormone will likely have impacts on other hormones as well. For example, changes in local auxin levels can affect at least five other plant hormones (Hansen and Grossmann, 2000; Fu and Harberd, 2003; Ponce et al., 2005; Rock and Sun, 2005; Raghavan et al., 2006). Perhaps the most widely studied relationship is that between auxin and cytokinins. These hormones tend to act antagonistically in plant development, with auxin promoting root development and cytokinin promoting shoot development (Coenen and Lomax, 1997). Alterations in the balance between these two hormones within a tissue will generally therefore re-direct the developmental program (Coenen and Lomax, 1997). Auxin exposure also stimulates ethylene production, high levels of which can, in turn, impair lateral and basipetal auxin transport in roots (Hansen and Grossmann, 2000). Exogenous application of auxin also leads to increased gibberellin synthesis (Fu and Harberd, 2003). The effect of auxin on brassinosteroid levels is unclear, although it is known that brassinosteroids appear to work in conjunction with auxin to promote the root gravitropic response, and that treatment of plants with either hormone impacts the transcriptional status of overlapping sets of genes (Kim et al., 2000; Nemhauser et al., 2004).

Finally, auxin has effects on ABA-mediated processes. As is the case with auxin and cytokinin, ABA and IAA tend to act antagonistically; for example, auxin promotes lateral root formation

and ABA inhibits it (De Smet et al., 2003). Furthermore, ABA exposure has been shown to lead to a decrease in the amount of free IAA in the treated plant (Dunlap and Robacker, 1990). With multiple hormones affecting similar biological functions, it is clear that the regulation of phytohormone signaling pathways must be very complex. One possible integrating centre for this signaling matrix is the previously mentioned SCF complex, since mutations in the different components of the complex affect multiple hormone sensitivities (ethylene, jasmonate, auxin and ABA), as well as creating defects in the response of the plant to environmental cues and developmental abnormalities (Woodward and Bartel, 2005). These are all outcomes that can be associated with alterations in either hormone production or sensing.

Since auxin qualifies as a mitogen, and MAPK signaling modules are ubiquitous across eukaryotic taxa, it should not be too surprising that regulation of auxin signaling also involves MAPKs. In fact, there is evidence for participation of at least two MAPK modules in auxin signaling (Mizoguchi et al., 1994; Kovtun et al., 1998; Mockaitis and Howell, 2000; Dai et al., 2006). First, an Arabidopsis MAPK module with an apparent negative impact on the auxin response has been reported that involves the MAPKKKs, ANP1/2/3, or, in tobacco, the corresponding orthologue, NPK1 (Kovtun et al., 1998). Ectopic expression of constitutively active variants of these MAPKKKs resulted in a dampened auxin response (Kovtun et al., 1998). Interestingly, the over-expression experiments also revealed positive roles for ANP/NPK1 in the oxidative stress response and it is known that oxidative stresses such as H_2O_2 can diminish auxin signaling (Kovtun et al., 1998; Kovtun et al., 2000). These results suggest that, in the presence of environmental stresses, auxin signaling may be suppressed. Further evidence for this idea is that over-expression of constitutively active NtNPK1 in transgenic tobacco conferred increased tolerance to heat, salt and cold stresses (Kovtun et al., 2000).

On the other hand, a second MAPK module may play a positive role in auxin signaling. Treatment of Arabidopsis seedlings with several auxins (20 μM NAA, 20 μM IAA, 20 μM 2,4-D), resulted in the rapid activation of a MAPK with a molecular size of ~44 kDa (Mockaitis and Howell, 2000), but this activation pattern was diminished in auxin-insensitive *axr4* mutants. This implies that MAPK activation in response to auxin requires a functional auxin sensing system, and is not the result of stress due to cytosolic acidification following exposure to high levels (300 μM) of 2,4-D, as claimed earlier (Tena and Renaudin, 1998). These Arabidopsis results were consistent with previous work which found that treatment of suspension-cultured tobacco BY-2 cells with 1 μM 2,4-D leads to MAPKK activation (Mizoguchi et al., 1994). Interestingly, application of the MAPKK inhibitor PD098059 was able to block auxin-induced expression of the gene encoding the 44 kDa MAPK, but had no effect on catalytic activation of the MAPK protein (Mockaitis and Howell, 2000). It would appear that two independent MAPKKs might be responsible for the gene activation signal and for phosphorylating the 44 kDa MAPK, but it should be noted that the ability of PD098059 to efficiently inhibit the activity of all plant MAPKKs has not been established (Mockaitis and Howell, 2000).

Abscisic acid

Abscisic acid (ABA) and ABA-induced signaling have been implicated in seedling, root and seed development, and in seed germination, as well as dehydration tolerance and general abiotic stress responses (Finkelstein et al., 2002; Himmelbach et al., 2003; Gubler et al., 2005; Roelfsema and Hedrich, 2005; Verslues and Zhu, 2005). Of particular importance to my project is the well-established association between ABA signaling and the control of the response of the plant to saline and drought conditions (Zhu, 2002; Deak and Malamy, 2005). Because of their environmental and commercial implications, these responses have been one of the most widely

studied aspects of ABA signaling. Many of these studies have focused on the response of the plant to externally applied ABA (Finkelstein et al., 2002; Yoshida et al., 2002; Takahashi et al., 2004). Plants respond to exogenous ABA in a number of ways, including closure of stomatal guard cells, and inhibition of lateral root formation (Finkelstein et al., 2002; Himmelbach et al., 2003; Gubler et al., 2005; Roelfsema and Hedrich, 2005; Verslues and Zhu, 2005). It has also been suggested that failure of seeds to germinate in highly saline environments may be a consequence of ABA signaling in response to high salt (Finkelstein et al., 2002).

Induction of physiological responses to osmotic stress, or to exogenous ABA, requires participation of numerous metabolic pathways and molecular players. This is illustrated by the frequency with which ABA-related genes have been recovered from both targeted and seemingly unrelated genetic screens (reviewed in Finkelstein et al., 2002). Screens for mutants displaying altered sensitivity to ABA, either during germination or during seedling/root growth, for suppressors of gibberellin-deficiency mutations, for high salt or sugar tolerance or for plants with abnormal responses to other hormones (e.g. auxin, ethylene and BR) have directly identified no fewer than 40 independent ABA signaling-related genes. The proteins they encode include protein kinases, protein phosphatases, G-proteins, transcription factors, RNA binding proteins, ribosomal proteins and phospholipid-associated proteins (Finkelstein et al., 2002). Further analysis of some of these mutant lines, as well as transcriptional profiling of the response of wild-type plants to exogenous ABA, has dramatically expanded the set of ABA-associated genes to >2 000 genes (Finkelstein et al., 2002; Finkelstein and Gibson, 2002; Mahalingam et al., 2003; Takahashi et al., 2004; Raghavan et al., 2006).

The extensive range of processes and genes implicated in ABA signaling raises the question of how apparently specific and appropriate cellular responses can arise from simple changes in

ABA levels. The first means of imparting specificity to the ABA response may be associated with the mechanism of ABA sensing. All attempts to biochemically or genetically identify validated ABA-receptors have failed, but a series of elegant experiments compared the effects of applying combinations of microinjected caged ABA with the changes induced by externally applying ABA to stomatal cells (Allan et al., 1994). The results strongly suggested that ABA can be detected both inter- and intra-cellularly, and that the pattern of ABA-induced changes appeared to be specific to the location of ABA detection (Hamilton et al., 2000).

Other mechanisms for ensuring specific ABA-induced responses may be derived from the types of signaling agents activated by ABA. For example, ABA exposure is known to result in rapid changes in cytosolic Ca^{2+} levels. Calcium ion fluxes can generate unique intracellular signatures based on the frequency and amplitude of local Ca^{2+} concentration oscillations, as well as on spatially restricted changes that affect only a specific cellular location or compartment (reviewed in Schroeder et al., 2001; Finkelstein et al., 2002). Altered Ca^{2+} fluxes in the cell have been shown to influence specific signaling pathways, including MAPK modules (Samuel et al., 2000; Kurusu et al., 2005). Since MAPKs are known to be involved in salt, drought and cold signaling (Nakagami et al., 2005), there would seem to be a strong possibility that MAPK signaling and ABA signaling are functionally linked. Indeed, in Arabidopsis, AtMEKK1 is transcriptionally up-regulated following salt exposure and drought conditions (Covic et al., 1999), and recent work has placed MEKK1 upstream of both MKK1 and MKK2 in the response to salt and drought stress (Teige et al., 2004). Both of these MAPKKs lie upstream of MPK4 (Teige et al., 2004). Other work has shown that the well-characterized MAPKs, MPK3 and MPK6, are also both activated in response to osmotic stresses (Droillard et al., 2002). However, since neither MKK1 nor MKK2 has been shown to activate MPK3, it would appear that at least one other

MAPKK is involved in the plant response to these stresses. In addition to MPK3 and MPK6, gene expression data available in public microarray repositories shows that other MAPKs are transcriptionally up-regulated following exposure to salt and ABA, including *MPK7* (Genevestigator). The involvement of multiple MAPK modules in these signaling pathways is not surprising when one examines the response of yeast to salt stress. The immediate response of yeast to osmotic stress is activation of the Hog MAPK pathway, but global transcript profiling revealed that other, unrelated MAPK genes are up-regulated at later time points, including the yeast *MKK1/MKK2* genes that are involved in cell wall remodeling (Roberts et al., 2000). This presumably reflects that fact that one of the physiological outcomes of osmotic stress in yeast is a change in growth habit that requires cell wall remodeling (Roberts et al., 2000). It is perhaps analogous that, in Arabidopsis, ABA treatment and osmotic stress both lead to diminished growth and photosynthetic rates; i.e. substantial re-direction of growth-related processes.

Ethylene biology

Another of the stress-induced plant hormones is the gaseous hormone, ethylene. The biological effects of ethylene in plants are wide-ranging, including functions in germination, cell elongation, senescence, abscission, pathogen interactions and abiotic stress responses (Chen et al., 2005). Ethylene can be synthesized by the majority of plant tissues from the stepwise conversion of methionine to S-adenosyl-L-methionine (AdoMet) to 1-aminocyclopropane-l-carboxylic acid (ACC) and finally to ethylene (Chae and Kieber, 2005). The enzymes responsible for each successive conversion reaction are Ado-Met synthetase, ACC synthase (ACS) and ACC oxidase (ACO; Chae and Kieber, 2005). The reaction that represents a metabolic commitment to ethylene production, and is also the rate limiting step in ethylene synthesis, is catalyzed by ACS (Chae and Kieber, 2005). Both ACS and ACO are encoded by

multiple genes in Arabidopsis, whose genome includes nine ACS (Yamagami et al., 2003) and five ACO variants (Chae and Kieber, 2005). Temporally and spatially localized ethylene production, and hence, induction of specific ethylene-regulated downstream signaling events, may be controlled in part by the subcellular localization and temporal expression patterns of the ACS enzymes, since promoter:GUS reporter studies of the various ACS genes have revealed expression patterns specific to each ACS isoform (Tsuchisaka and Theologis, 2004). Other modes of controlling ethylene production include regulation of the abundance of ACS and ACO proteins by both transcriptional and post-translational processes. Numerous studies have reported that induction of expression of discrete *ACS* and *ACO* genes is treatment-specific (Abel et al., 1995; Chae et al., 2000; Tsuchisaka and Theologis, 2004; Wang et al., 2005). For example, auxin exposure triggers a burst of ethylene production that is preceded by specific up-regulation of *ACS4* expression (Abel et al., 1995). Further transcriptional control over ethylene biosynthesis can be dependent upon the overall hormone status of the plant, since rice *OsACO3* expression is up-regulated in the presence of ethylene, but not in conjunction with high levels of auxin (Chae et al., 2000),while *OsACO2* expression is up-regulated in the presence of auxin, but not if ethylene is also abundant in the cell (Chae et al., 2000).

It is now widely accepted that post-translational control of ethylene production involves the rapid turnover of ACS proteins mediated by the 26S proteasome (reviewed in Chae and Kieber, 2005). Phosphorylation of specific ACS proteins by either CDPKs or MAPKs prevents protein degradation by the proteasome, resulting in increased ethylene biosynthesis and, hence, induction of downstream signaling events (Tatsuki and Mori, 2001; Liu and Zhang, 2004; Sebastia et al., 2004).

Unlike the situation with some other plant hormones, ethylene receptors have been identified and well-characterized (Chen et al., 2005). The Arabidopsis genome encodes five ethylene receptors, of which at least three are situated on the membrane of the endoplasmic reticulum (Chen et al., 2005). Because ethylene is a gaseous hormone, it can freely diffuse across the plasma membrane into the cytosol, which precludes a specific requirement for an extracellular ethylene receptor (Chen et al., 2005). There also appears to be no requirement for a full complement of functional ethylene receptors, since single knock-out mutants for each receptor do not display abnormal phenotypes (Hua and Meyerowitz, 1998).

Analysis of combinatorial loss-of-function and independent gain-of-function mutants indicates that ethylene receptors are negative regulators of ethylene signaling. In the absence of ethylene, the receptors interact with, and maintain activity of, the immediate downstream effector protein CTR1 (Huang et al., 2003). *CTR1* encodes a MAPKKK which, in its active state, also functions as a negative regulator of ethylene responses (Huang et al., 2003). Current hypotheses postulate that interaction between the "empty" ethylene receptor and CTR1 disrupts the association of the N-terminal auto-inhibitory domain of CTR1 with its kinase domain, thereby keeping the MAPKKK in its active state (Huang et al., 2003). Binding of ethylene to the receptor causes a conformational change in the receptor that interferes with this receptor-CTR1 interaction, which allows the CTR1 auto-inhibitory domain to silence the MAPKKK and releases the downstream steps from CTR1-dependent inhibition. To date, neither a MAPKK nor a MAPK downstream of CTR1 have been identified through mutant screens, which suggests that loss of either component is either lethal or compensated for through redundancy. However, such screens have identified other downstream effectors including EIN3 and ERF1 (Stepanova and Alonso, 2005).

Independent of the CTR1 ethylene-sensing module, two MAPKKs and one MAPK have been

identified that promote ethylene biosynthesis, hence, contribute to ethylene-mediated signaling. These include the tobacco orthologue of AtMKK4, NtMEK2, and Arabidopsis AtMKK9, both of which appear to activate MPK6 or its tobacco orthologue, SIPK (Kim et al., 2003; Cluis, 2005). MPK6, in turn, promotes ethylene biosynthesis by phosphorylating ACS2 and ACS6 on multiple C-terminal serine residues (Liu and Zhang, 2004), which prevents degradation of the ACS protein by the 26S proteasome and thus results in increased ethylene biosynthesis (Liu and Zhang, 2004).

Jasmonate biology

Several long-chain fatty acid derivatives (octadecanoids, or oxylipins) have been shown to act as signal molecules in plants (Liechti and Farmer, 2006). Jasmonic acid and methyl jasmonate (MeJA) are the most widely studied octadecanoid derivatives, but other jasmonate precursors and derivatives such as OPDA and Z-jasmonate have also been shown to have biological activity (Liechti and Farmer, 2006). Collectively, these function in several areas of plant physiology including the mediation of stress-responses and regulation of development and metabolism (Liechti and Farmer, 2006). In the context of this review, unless otherwise specified, jasmonate refers to the net effect of all such jasmonate-related metabolites. Studies of jasmonate signaling have primarily focused on the role of jasmonate in the stress-and wound-response pathways. It has been well documented that wounding of plant tissues triggers jasmonic acid biosynthesis (Lorenzo and Solano, 2005; Schilmiller and Howe, 2005) and several gene expression profiling experiments have demonstrated that these increases in jasmonic acid content are correlated with large-scale expression changes (Devoto et al., 2005; Sasaki-Sekimoto et al., 2005; Taki et al., 2005). In general, it appears that jasmonate-induced changes serve a protective role by increasing the ability of the plant to withstand both biotic and abiotic stresses (Li and Zhang,

2002; Tuominen et al., 2004). Consistent with this, several jasmonic acid biosynthesis and sensing mutants are generally more susceptible to damage from environmental stresses, with *fad, jar1-1* (*jasmonic acid resistant 1-1*) and *coi1-1* (*coronatine insensitive 1-1*) showing ozone hypersensitivity and increased pathogen susceptibility (Berger, 2002).

It is not clear, however, which signaling pathways are specifically activated in response to jasmonate. Jasmonate-induced changes include altered secondary metabolite production, such as increased synthesis of anti-predatory terpenes and proteinase inhibitors (Brader et al., 2001; Schilmiller and Howe, 2005). Other jasmonate-induced changes include increased sulfur metabolism, and the activation of the glutathione and ascorbate pathways involved in controlling the antioxidant status of the cell (Sasaki-Sekimoto et al., 2005). Because MAPK signaling is intricately linked to oxidative stress responses (Kovtun et al., 2000; Samuel et al., 2000; Samuel and Ellis, 2002; Tuominen et al., 2004; Miles et al., 2005), it seems likely that jasmonate-induced changes in the antioxidant status of the cell will involve MAPKs. Although jasmonate-mediated responses to mechanical and abiotic stresses are similar (reviewed in Browse, 2005), for the purposes of this thesis, only the environmental and specifically oxidative stress-induced, jasmonate-mediated processes will be discussed further.

Exposure to oxidative stresses such as ozone at levels capable of initiating cell death formation also induces jasmonate production (Sasaki-Sekimoto et al., 2005), a response which in turn results in a heightened antioxidant status in the cell, as reflected in increased ascorbate levels (Sasaki-Sekimoto et al., 2005). Consistent with this, both jasmonate and ascorbate biosynthetic mutants such as *opr3* and *vtc1* respectively, are hyper-sensitive to ozone (Conklin et al., 2000; Sasaki-Sekimoto et al., 2005). Little is known regarding the intermediate signaling events invoked by jasmonate that enable oxidative protection, and to date no jasmonate receptors have

been identified. The jasmonate sensing locus, *COI1*, is crucial for cell survival following oxidative stress (Lorenzo et al., 2004; Devoto et al., 2005) since *coi1-1* mutants, are also hypersensitive to ozone. While it does not appear that COI1 is a jasmonate receptor, it is an F-box protein that forms a component of SCF-type E3 ubiquitin ligase that targets repressors of JA signaling for degradation (Devoto et al., 2005). Additionally, COI1 is closely related to the auxin receptor TIR1 (Lorenzo and Solano, 2005) based on sequence homology, indicating that further characterization of the function of COI1 is necessary. It also appears that the plant's response to jasmonate involves the activation of multiple MAPK signaling modules (Petersen et al., 2000; Lampard et al, unpublished). Wounding leads to activation of Arabidopsis MPK4, and *mpk4* plants fail to show MeJA-induced induction of *PDF1.2* and *THI2.1,* two known jasmonate-responsive genes (Petersen et al., 2000). More recently, the tobacco orthologue of MPK4, NtMPK4, was found to be required for proper induction of some, but not all, jasmonate responsive genes (Gomi et al., 2005). The same study showed that NtMPK4 is rapidly activated by ozone exposure and that *NtMPK4* loss-of-function mutants are hypersensitive to ozone, perhaps as a result of jasmonate insensitivity (Gomi et al., 2005). Hence, it appears that MPK4 activity is needed for a complete jasmonate response. However, it is also clear that MPK4 signaling is not exclusive to the classical jasmonate responses, since *mpk4* mutants also accumulate salicylate, display constitutive systemic acquired resistance (SAR) and are more resistant to the pathogens *Pseudomonas syringae* pv. *tomato* and *Peronospora parasitica* (Petersen et al., 2000). Recently, MAP kinase 4 substrate 1 (MKS1) was identified as a substrate of activated MPK4, (Andreasson et al., 2005). The function of this protein is unknown but over-expression of *MKS1* in Arabidopsis confers a semi-dwarf phenotype with increased resistance to infection by *Pseudomonas syringae* pv. *tomato* DC3000 (Andreasson et al., 2005). Although

MKS1 is a substrate of MPK4, MKS1 does not appear to be involved directly in the wounding or jasmonate responses since the transcript profiles of wound and jasmonate induced genes such as *PDF1.2* which are mis-regulated in *mpk4*-null mutants are not affected in *MKS1* over-expression or RNAi-silenced plants (Andreasson et al., 2005). At least two other MAPKs have been shown to be activated by the same stresses that activate MPK4; both MPK3 and MPK6 are activated in response to wounding, pathogen infection and oxidative stresses, including ozone (Ichimura et al., 2000; Samuel et al., 2000; Droillard et al., 2002; Samuel and Ellis, 2002; Miles et al., 2005). The tobacco orthologue of MPK3 (WIPK) has been shown to be involved in jasmonate metabolism/signaling in tobacco, since co-suppression of WIPK blocked jasmonate formation in response to wounding (Seo et al., 1995). That each of MPK3, MPK4 and MPK6 are activated in response to treatments known to invoke jasmonate biosynthesis suggests that MAPK signaling modules including MPK3, MPK4 and MPK6 function in jasmonate-induced signaling. Whether other MAPKs are involved in these processes remains to be seen. Identification of the upstream, activating MAPKKs will prove insightful in determining the precise functions each MAPK has in these signaling modules.

Salicylate biology

Salicylic acid is also involved in plant stress- and defense-responses and has crucial functions in the response to pathogen infection and oxidative stresses (Alvarez, 2000). Similar to jasmonate, several functional salicylate derivatives exist in plants, including salicylate-glycosides and the volatile ester, methyl-salicylate (Alvarez, 2000). However, most studies involving salicylate quantification refer to either the free salicylate pool, or the combination of salicylate and salicylate-glycoside levels (total SA) within the cell (Alvarez, 2000).

Most evidence suggests that salicylate functions antagonistically to jasmonate in plants (Tuominen et al., 2004; Beckers and Spoel, 2006; Mur et al., 2006). Plant responses to application of exogenous salicylate have been reported to include changes in ion transport, stomatal closure, changes in both growth and photosynthetic rates, altered floral development and changes in the antioxidant status of the cell (Alvarez, 2000; Schroeder et al., 2001; Shah, 2003; Dong, 2004; Roelfsema and Hedrich, 2005; Wiermer et al., 2005; Mur et al., 2006). It should be noted that these responses can be concentration-dependent and it is not known whether all of them necessarily occur in response to physiological (endogenous) levels of salicylate. On a molecular level, the best characterized response to salicylate treatment is a marked increase in expression of acidic pathogenesis-related (PR) genes, and accumulation of the encoded proteins (Ryals et al., 1996; Zhang and Klessig, 1997; Alvarez, 2000), but it is also associated with induction of protein phosphorylation, including the rapid activation of MAPKs (Zhang and Klessig, 1997). Because of the relationship between SA and disease resistance during plant-pathogen interactions, much of the research on SA in plants has focused on this role (reviewed in Wiermer et al., 2005; Mur et al., 2006). Upon pathogen infection of a resistant plant by an avirulent pathogen, the plant characteristically displays the hypersensitive response (HR), defined largely by programmed-cell death that results in the formation of lesions surrounding the point of infection (Nimchuk et al., 2003) . Prior to visible lesion formation, salicylate accumulates in the responding tissue in a biphasic manner (Malamy et al., 1990). If the first wave of salicylate accumulation is blocked, the appearance of lesions resulting from TMV infection is delayed, but the eventual lesion size increases, as does the dispersal of virions (Mur et al., 1997), which suggests that the initial peak of SA accumulation is required for rapid appearance of lesions and limiting both the eventual size of the lesion and viral dispersal.

However, the precise signaling events occurring downstream of this initial SA burst have yet to be defined.

The HR is also known to induce SAR, which enables the plant to be more resistant to further pathogen infection. Several findings indicate that salicylate plays a role in both of these processes. First, transgenic plants over-expressing a bacterial salicylate hydroxylase (NahG) which breaks down salicylate and prevents its accumulation within the cell, fail to display lesion formation and SAR upon pathogen infection (Gaffney et al., 1993). Furthermore, plants with elevated levels of salicylate show increased resistance to pathogens, constitutive expression of PR genes and occasionally show spontaneous HR-like lesion formation (Bowling et al., 1997). Finally, pre-treatment of plants with salicylate confers increased resistance to plant pathogens (Shah, 2003). A role for salicylate in both triggering or promoting cell death via lesion formation while concurrently serving a defensive function by imparting heightened resistance to further pathogen infection, might appear contradictory. However, a concentration gradient of salicylate exists between the centre of the lesion and cells on the periphery, with the highest concentration at the centre of the lesion (Enyedi et al., 1992). It has been proposed that the modest elevation of salicylate concentrations in the periphery, perhaps in conjunction with the jasmonate levels in these cells, promotes SAR, while the very high salicylate concentrations within the lesion trigger cell death.

At least three MAPK modules function downstream of pathogen infection. One of these has been defined from receptor to effector, and is activated by the bacterial elicitor, flagellin 22 (flg22; Asai et al., 2002). In Arabidopsis, recognition of the flg22 peptide by the FLS receptor, results in the sequential activation of MEKK1, MKK4/5 and MPK3/6, which results in activation of WRKY22 and WRKY29 transcription factors (Asai et al., 2002). A similar MAPK module

appears to operate in tobacco where the AtMPK3 and AtMPK6 orthologues, WIPK and SIPK are activated by tobacco mosaic virus infection (Zhang and Klessig, 1998). Furthermore, activation of NtMEK2, the orthologue of AtMKK4/5 leads to HR-like cell death (Jin et al., 2003).

A second MAPK pathway has been detected in tobacco that is responding to infection by tobacco mosaic virus (TMV) infection (Liu et al., 2004). TMV infection triggers the activation of a MAPK module that includes the MAPKKK, NtNPK1, the MAPKK, NtMEK1, and the MAPK, NtNtf6 (Liu et al., 2004). The fact two apparently distinct pathways are activated in response to TMV infection is intriguing. This could reflect redundancy in the response to pathogens, or at least TMV infection. However, because disruption of either pathway leads to increased susceptibility to TMV infection, it appears that that each pathway has specific, critical functions in N-mediated resistance. Finally, a third MAPK module appears to function as a negative regulator of SAR and salicylate biosynthesis, since Arabidopsis *mpk4* mutants display heightened salicylate concentrations, constitutive PR genes expression and SAR (Petersen et al., 2000).

Oxidative stresses such as acute ozone exposure also induce localized cell death that is manifested in the formation of HR-like lesions (Samuel et al., 2000; Ogawa et al., 2005). It appears that salicylate and salicylate-induced signaling also functions in the development of these lesions, since suppression of SA accumulation in NahG transgenic tobacco plants renders the plants less susceptible to ozone-induced lesion formation (Orvar et al., 1997; Ogawa et al., 2005). Like salicylate and jasmonate signaling, salicylate and ethylene signaling appear to interact as well. Ozone exposure of Arabidopsis and tobacco plants results in increased salicylate accumulation within the exposed tissue and this appears to be controlled in part by an

ethylene burst that occurs soon following ozone exposure (Ogawa et al., 2005). Given the involvement of MAPK signaling with jasmonate and ethylene signaling, it is not surprising that MAPKs also appear to influence the interactions with salicylate as well. One of the earliest events following ozone exposure is the activation of the MAPKs, SIPK and WIPK, in tobacco (Samuel et al., 2000), and of the respective Arabidopsis orthologues, AtMPK6 and AtMPK3 (Miles et al., 2005). SIPK (originally identified as salicylate-induced protein kinase) activation appears to promote HR-like lesion formation, possibly by triggering increased ethylene production, but it may also have a negative effect on salicylate signaling, since ozone-induced salicylate accumulation is blocked in SIPK-over-expressing plants (Samuel et al., 2005). Although the mechanisms underlying salicylate-induced lesion formation have yet to be completely characterized, it is clear that these processes involve modulation of MAPK signaling modules and interplay with at least two other hormones, namely jasmonate and ethylene.

ARABIDOPSIS MKK3

The contrast between the relatively small number of Arabidopsis MAPKKs and the larger families of upstream MAPKKKs and downstream MAPKs suggests that individual MAPKKs may play particularly crucial roles within the MAPK signaling matrix *in planta*. Among the MAPKKs, AtMKK3 stands out because of its unique protein architecture, long evolutionary history and lack of any paralogues in the plant genomes sequenced to date. The original report of the cloning of the AtMKK3 cDNA showed that the gene is expressed in roots, leaves, stems and flowers (Ichimura et al., 1998), and examination of microarray databases shows that MKK3 is almost universally transcribed in plant tissues. However, no mutants of this locus have been recovered from forward genetic screens, and the only functional information is an earlier report

that MKK3 appears to interact with AtMPK1 in a yeast two-hybrid screen (Ichimura et al., 1998).

The MKK3 protein includes a canonical MAPK docking site and the dual-specificity Ser/Thr-Tyr kinase domain characteristic of all MAPKKs. However, it is unusual in containing an extended C-terminal NTF2 domain, the function of which is currently unknown. In yeast, NTF2 proteins play a central role in the nucleo-cytoplasmic shuttling of proteins, and NTF2-deletion mutants are lethal (Stewart, 2000). Yeast NTF2 mediates the import of Ran-GDP into the nucleus where it is rapidly converted to the Ran-GTP form (Quimby et al., 2000). A concentration gradient of Ran-GTP between the nucleus and the cytosol is required to facilitate the export of proteins in the form of a Ran-GTP/carrier protein complex (Quimby et al., 2000). However, NTF2 is also required for maintenance of proper nuclear import pathways, since cycling of importin-β, a carrier of imported proteins containing nuclear localization sequences, requires a constant supply of Ran-GTP in the nucleus (Quimby et al., 2000).

Aside from MKK3, a small number of other NTF2 domain-containing proteins have been identified, including *S. pombe* Mex67 and human TAP, both of which have been shown to be involved in nuclear export of mRNA in an NTF2 domain-mediated fashion (Thakurta et al., 2004). However, none of these other NTF2 domain-containing proteins also possess protein kinase domains. While Arabidopsis, like other eukaryotes, possesses canonical NTF2 homologues (Zhao et al., 2006), it does not appear that any other proteins encoded in the Arabidopsis genome contain NTF2-domains. Of the three separate NTF2-like proteins encoded in the Arabidopsis genome, at least two appear to control nuclear import of Ran-GDP in plants, and to be required for maintenance of proper nuclear transport processes (Zhao et al., 2006).

Clearly, deciphering the role of the NTF2 domain in MKK3 would provide important insights into the biological functions of this dual-function MAPKK in Arabidopsis and other plants. Database searches reveal that putative orthologues of MKK3 exist in tobacco, poplar, parsley, *Suaeda maritima* subsp. *salsa*, rice, *Selaginella moellendorffii* and *Chlamydomonas* (Figure 1.1). Interestingly, the only MAPKK encoded in the *Chlamydomonas* genome appears to be the putative MKK3 orthologue (Hamel et al., 2006). Since *Chlamydomonas* is generally regarded as reflecting many of the characteristics of the hypothetical common ancestor to all plant species, this would imply that all plant MAPKKs share this ancient MKK3 homologue as a common ancestor. Deciphering the biological function of MKK3 signaling would therefore be of broad interest to the plant research community.

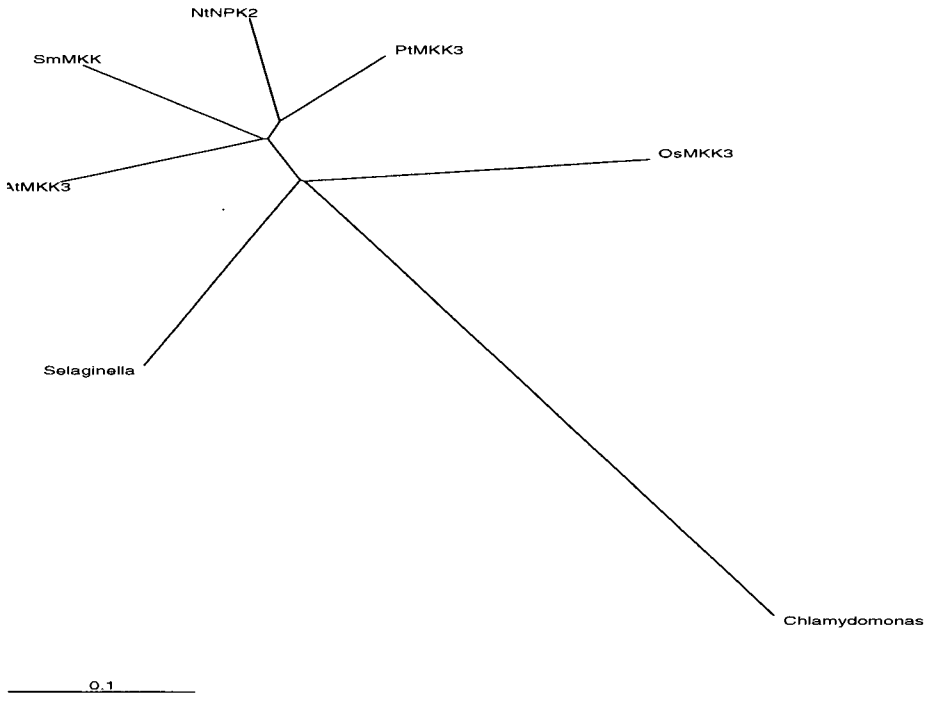

Figure 1.1. Un-rooted phylogenetic analysis of group B MAPKKs. Sequences were identified in the following organisms: Arabidopsis (AtMKK3), Poplar (PtMKK3), Tobacco (NtNPK2), Rice (OsMKK3), *Suaeda maritima* subsp. *salsa* (SmMKK3), *Selaginella moellendorffii* (Selaginella) and *Chlamydomonas*. It appears that dicot group B MAPKKs have diverged significantly from the more distantly related monocot, lycophyte and chlorophyte group B MAPKKs.

CONCLUSIONS AND OBJECTIVES

Several lines of evidence indicate that signal transduction pathways operate less as linear sequences so much as in a matrix or network within which extensive crosslinks operate between signaling modules. In terms of stress signaling in Arabidopsis, it is clear that MAPK activation and hormone biosynthesis are intertwined, while analysis of the MAPK module gene families suggests that one important level of signal integration is likely to occur at the level of the MAPKKs. The objectives of this research program were to examine MAPKK signaling in Arabidopsis, focusing on the phylogenetically unique MAPKK, MKK3. This was carried out by:

1. Characterizing the expression profile of MKK3.
2. Examining the phenotypic consequences of modifying MKK3 expression in the plant.
3. Identifying genes for which the expression is controlled/influenced by MKK3.
4. Identifying substrates/interacting partners of MKK3.
5. Examining the function of the NTF2 domain of MKK3.

In this thesis I describe the results of a series of global transcriptional profiling and biochemical approaches that were used to identify potential roles for the previously uncharacterized MAPKK, MKK3. These experiments revealed that MKK3 signaling may be linked to multiple hormone and stress responsive signaling pathways, possibly in an NTF2 domain-mediated fashion, and that it could act through the MAPKs, MPK1, MPK2 and MPK7.

CHAPTER 2. ANALYSIS OF *MKK3* EXPRESSION

INTRODUCTION

The Arabidopsis genome encodes >60 MAPKKKs, 10 MAPKKs and 20 MAPKs (Ichimura et al., 2002; Hamel et al., 2006). The convergence at the MAPKK level suggests that these kinases could be important mediators and integrators between signaling modules, yet the majority of MAPK research conducted to date has focused on the MAPK level (Jonak et al., 2002; Nakagami et al., 2005; Pedley and Martin, 2005). Phylogenetic analysis of plant MAPKKs indicates that there are four distinct classes of MAPKKs and that MKK3 is the only Arabidopsis group B MAPKK (Ichimura et al., 2002; Hamel et al., 2006). Group B MAPKKs are defined by the presence of an extended C-terminus that encodes a nuclear transport factor 2 (NTF2) domain, the function of which is currently unknown (Ichimura et al., 2002). Putative MKK3 orthologues have been cloned from both monocot and dicot plant species, including poplar, rice and tobacco, but the biological functions of this kinase have yet to be determined (Shibata et al., 1995; Ichimura et al., 1998; Ichimura et al., 2002; Hamel et al., 2006). A single group B MAPKK can also be found in the lycophyte, *Selaginella moellendorffii*, (http://selaginella.genomics.purdue.edu./) indicating that these MAPKKs functioned in some of the earliest vascular plants. Interestingly, basal plant species such as *Chlamydomonas* possess a single MAPKK which also belongs to group B (Hamel et al., 2006). While *Chlamydomonas* is not thought to represent a direct ancestor of vascular plants, it clearly diverged from the common ancestor of the modern Chlorophytes and Embryophytes at a very early point in plant evolution. Thus, the presence of only a group B MAPKK suggests that all plant MAPKKs could possibly be derivatives of an ancestral group B MAPKK. Furthermore, the fact that NTF2 domain-

containing MAPKKs are used by both lower- and higher-order plants suggests that these MAPKKs may be involved in conserved and essential cellular processes; for example, homeostasis, cell division or environmental responses. Even with the implied importance of group B MAPKKs, the only functional data available for MKK3 and its orthologues are gene expression data (Mizoguchi et al., 1994; Ichimura et al., 1998; Hamel et al., 2006) Genevestigator). AtMKK3 was initially cloned by Ichimura et al (1998), who noted that *MKK3* expression occurs in roots, leaves, stems and flowers (Ichimura et al., 1998). Gene expression patterns can be important indicators of possible function, but each of the organs examined by Ichimura *et al* contains several cell types. I therefore undertook a higher resolution analysis of *MKK3* expression, in anticipation that the resulting data could be used to formulate hypotheses regarding the function of putative MKK3 signaling modules.

The flexibility and ease of gene expression analysis using promoter-GUS reporters made this an attractive system for the study of *MKK3* expression patterns. Using a *MKK3 promoter:GUS* construct, and histochemical staining for GUS expression, *MKK3* promoter activity was examined throughout the Arabidopsis developmental cycle, from germination to maturity. Analysis of *MKK3* expression in response to a comprehensive treatment panel designed on the basis of CAREs situated in the *MKK3* promoter sequences was also carried out.

MATERIALS AND METHODS

In silico analysis of *MKK3* promoter sequences

The internet-based algorithms PLACE (http://www.dna.affrc.go.jp/PLACE/) and Athena (http://www.bioinformatics2.wsu.edu/cgi-bin/Athena/cgi/home.pl) were used to identify the presence of known CAREs in the *MKK3* promoter sequences (Higo et al., 1999; O'Connor et al.,

2005). These tools scan a query sequence and generate a list of CAREs contained within the query sequence, based on comparison with a database of published transcription factor binding motifs. Preliminary analysis was completed in PLACE by querying the 1500 base pairs upstream of the *MKK3* start codon; following the release of Athena, the same sequence was re-analysed using a p-value cutoff of 10^{-1}. In the context of single promoter analysis, Athena provides a statistical report that indicates the likelihood that a given CARE is over-represented in the promoter relative to the frequency of occurrence of the same CARE in the rest of the genome.

Transgenic Arabidopsis plants expressing an *MKK3 promoter:GUS* reporter construct were subjected to a panel of external stimuli designed on the occurrence of CAREs predicted by Athena to be over-represented in the *MKK3* promoter sequences (p-value <0.05). The responsiveness of the promoter to these stimuli was determined by analyzing resultant GUS activity.

In silico analysis of *MKK3* expression patterns

The expression pattern of *MKK3* was examined in both the Arabidopsis Massively Parallel Signature Sequencing (MPSS) database (http://mpss.udel.edu/at/) and the publicly available microarray datasets (Genevestigator; https://www.genevestigator.ethz.ch/). MPSS detects the abundance of transcripts by sequencing unique 17-20 base-pair tags (signatures) generated by restriction digestion of cDNA with *Dpn*II (Nakano et al., 2006). The frequency of occurrence of each tag in a given library is recorded and used to generate an expression profile for each specific tag. Expression profiles for each Arabidopsis MAPKK were obtained from the Arabidopsis MPSS site; the data obtained from these analyses represent the sum of abundances for all signature sequences specific to each gene.

MKK3 expression data in the publicly available Arabidopsis microarray datasets were examined using Genevestigator. Genevestigator was originally released to the public in 2004, but has since been updated (Zimmermann et al., 2005), and the current release allows access to over 1800 Affymetrix-based full genome microarray datasets. The complete collection of microarray datasets was queried using default settings to obtain expression data for each MAPKK gene in different tissue types, developmental stages, and mutant lines, and in response to a wide range of exogenous treatments (Zimmermann et al., 2005).

Construction of *pMKK3PR:GUS* reporter construct

The plasmid *pMKK3PR:GUS* contains the *beta*-glucuronidase (GUS) reporter gene placed under the control of *MKK3* promoter sequences (Figure 2.1). The intergenic region between the *MKK3* coding sequence (At5g40440) and the coding sequence of the neighbouring gene (At5g40430) comprises 3950 base pairs and the two coding sequences lie in a head-to-head pattern, suggesting that their 5'-regulatory regions might overlap to some degree. Therefore, to minimize this possibility, the 1500 base pair region upstream of the start codon of *MKK3* was arbitrarily defined as the *MKK3* promoter sequence.

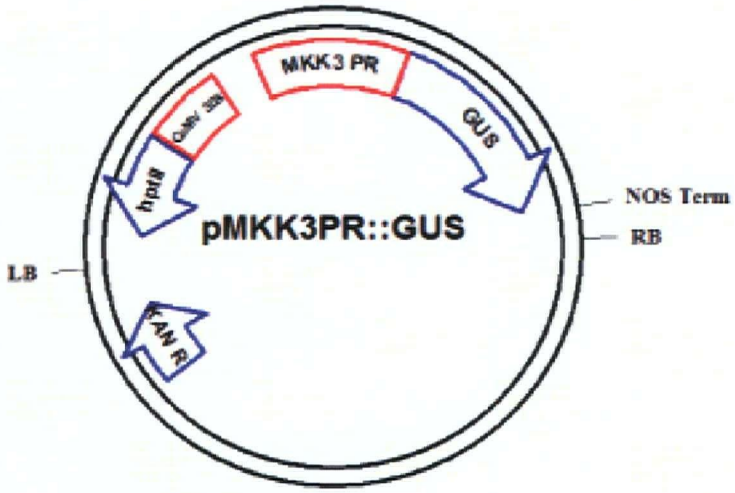

Figure 2.1: pMKK3PR:GUS. 1500 base pairs proximal to the start codon of *MKK3* were isolated and cloned upstream of sequences encoding the β-glucuronidase (GUS) reporter situated in the parent binary vector *pCAMBIA1381Z.*

These sequences were isolated from WT genomic DNA by PCR amplification (Appendix 1) using Platinum Taq HIFI (Invitrogen, Burlington, ON, Canada) and KK3PRF (5' CGG AAT TCG ACT TGA CAC TTT ATG AGT 3') and KK3PRR (5' CGG GAT CCG ATA ACT TTT TCT GTA ACA CAG 3') primers. After amplification, *MKK3* promoter sequences were subcloned into the cloning vector *pCR2.1* (Invitrogen, Burlington, ON, Canada) via TOPO TA-mediated cloning (Appendix 1) creating the intermediate plasmid *pCR2.1-MKK3PR.* The integrity of subcloned promoter sequences was verified by DNA sequencing. They were subsequently excised from *pCR2.1-MKK3PR* by restriction digestion with *EcoRI* and *BamHI,* and isolated via gel purification using the QiaQuik Gel Purification Kit (Qiagen, Mississauga, ON, Canada). Purified promoter sequences were then cloned into the binary vector *pCAMBIA1381Z,* immediately upstream of the *GUS* reporter gene (Appendix 1). Prior to transformation of Arabidopsis plants, the integrity of the *MKK3* promoter sequences in

pMKK3PR:GUS was verified by DNA sequencing at the Nucleic Acid and Protein Services (NAPS) facility at UBC. (Appendix 1).

Generation of transgenic Arabidopsis plants expressing a *MKK3 promoter:GUS* reporter

The *pMKK3PR:GUS* binary vector was introduced into Arabidopsis plants via the floral dip method (Clough and Bent, 1998). Briefly, a 250 mL culture of *Agrobacterium tumefaciens* EHA105 carrying the *pMKK3PR:GUS* construct was grown to saturation while shaking at 200 rpm (28°C). Bacterial cells were collected by centrifugation at 4000 x g for 15 minutes, and resuspended in 200 mL 5% sucrose containing 0.05% Silwet L-77. Flowering Arabidopsis thaliana "Columbia 0" plants with approximately 5 cm bolts were dipped into the *A. tumefaciens* suspension for five seconds. Dipped plants were held in plastic bags in the dark for a period of 48 hours prior to their transfer back to normal growth chamber conditions for a period of seven days. The same plants were then dipped a second time using a freshly prepared *A. tumefaciens* suspension. After the second dipping process, plants were returned to normal growth conditions until seeds were set.

Mature T1 seeds were harvested and transformants were recovered by selection of germinating seedlings on ½ MS plates (Appendix 2) containing 35 μg/mL hygromycin B. Individual rosette leaves from antibiotic-resistant T1 plants were screened for expression of *GUS* sequences via histochemical analysis of GUS activity, and *GUS*-expressing T1 plants were allowed to grow until seed set. T2 seeds that germinated in the presence of 35 μg/mL hygromycin B were transferred to soil and cultivated until seed set. Seeds from individual T2 plants were collected for analysis in the T3 generation for segregation of hygromycin B resistance to identify plants homozygous for the T-DNA insertion carrying the *MKK3 promoter:GUS* fusion sequence. GUS

activity in homozygous *MKK3 promoter:GUS* T3 plants was then analyzed throughout development and in response to a panel of hormone and abiotic stresses as described below.

Histochemical analysis of GUS activity

The histochemical protocol developed by Jefferson (1987) and the fixation method developed by Malamy and Benfey (1997) were used to monitor GUS activity in the *MKK3 promoter:GUS* plants (Jefferson, 1987; Malamy and Benfey, 1997). Histochemical detection of GUS gene expression is based on cleavage of a synthetic exogenous substrate, 5-bromo-4-chloro-3-ondoyl-glucuronide (X-gluc), by GUS, which results in the deposition of a blue precipitate at the sites where the protein is being expressed (Jefferson, 1989). Transgenic plants were subjected to heptane treatment to allow for penetration of staining solution into host tissue in 12-well tissue culture plates (Fisher Scientific, Nepean, ON, Canada) for ten minutes, after which the heptane was aspirated. Residual solvent was allowed to evaporate for a period of five minutes. Afterwards, samples were flooded with GUS staining solution (0.5 mg/mL X-gluc; 0.1% v/v Triton X-100; 0.25 mM $K_4Fe(CN)_6 \bullet 3H_2O$; 0.25 mM $K_3Fe(CN)_6$; 50 mM sodium phosphate buffer, pH 7.0) and cell culture plates were covered and placed in the dark at 37°C for 8 hours. Excess staining solution was removed by aspiration and the tissue samples were cleared by incubation for 15 minutes at 57°C in 20% methanol containing 0.25N HCl. This solution was then replaced with 60% ethanol containing 7% NaOH and samples were incubated for 15 minutes at 20°C. This solution was replaced sequentially with 40%, 20% and 10% ethanol solutions, with the samples being incubated in each re-hydration solution for five minutes at 20°C. Cleared, re-hydrated samples were then placed in storage buffer (5% ethanol; 20% glycerol) and GUS activity was recorded photographically.

Treatment of transgenic Arabidopsis plants expressing the *MKK3 promoter:GUS* construct

Unless otherwise specified, all treatments of *MKK3 promoter:GUS*-expressing plants were carried out using 14-day-old plants cultivated on ½ MS plates. Plants were transferred from ½ MS plates to ½ MS plates containing the appropriate additives (treatment or control) and maintained on these plates for a period of 24 hours, following which histochemical analysis of GUS activity was performed. In all cases, control plants were also transferred to fresh plates to ensure that handling of the plants had not resulted in changes in *MKK3* promoter activity. Treatments and control additive preparation are described in Appendix 2.

RESULTS

Examination of MKK3 promoter sequences

The Arabidopsis genome architecture upstream of the gene encoding MKK3 (At5g40440) is such that the adjacent gene (At5g40430) lies 3950 base pairs upstream of MKK3 and its open reading frame lies on the opposite chromosome strand (3' to 5'). In addition, the first intron of the MKK3 gene is contained within the 5' untranslated region (UTR), upstream of the MKK3 translational start codon (Figure 2.2). Examination of the complete intergenic region, using PLACE, revealed that the majority of predicted CAREs were located within 1500 base pairs of the MKK3 translation initiation codon (Figure 2.3). Because of this, and the fact that many CAREs are found within the first intron of plant genes (Zhang et al., 1994; Gidekel et al., 1996; de Boer et al., 1999; Dorsett, 1999), the 1500 base pairs proximal to the start codon of the gene encoding MKK3 were used for further *in silico* and *in vivo* promoter analysis.

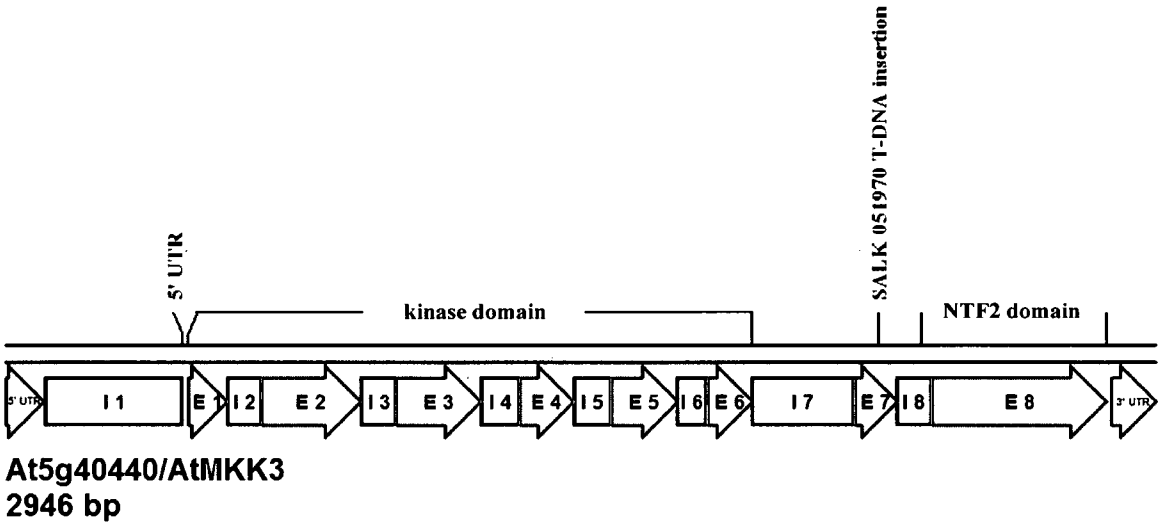

Figure 2.2: At5g40440/MKK3 gene architecture. The genomic DNA encoding MKK3 spans 2946 base pairs and is comprised of 8 introns and exons. The start codon is situated 14 base pairs downstream of the 3'-end of the first intron, with all remaining upstream sequences comprising 5'UTR. The dual-specificity kinase domain is encoded by exons 1-6 and the NTF2 domain is encoded by exon 8. The T-DNA insertion in the SALK 051970 T-DNA insertional mutant line lies in exon 7, which is located between the kinase and NTF2 domains.

In silico analysis of *MKK3* promoter sequences

Analysis of the MKK3 1500bp promoter in PLACE and Athena provided similar results and both identified several hormone and stress-response cis elements, as well as a putative cell cycle box (Figure 2.3). These findings were used to guide experiments in which I examined the behaviour of the *MKK3* promoter in response to treatment with various hormones, and to abiotic and biotic stresses.

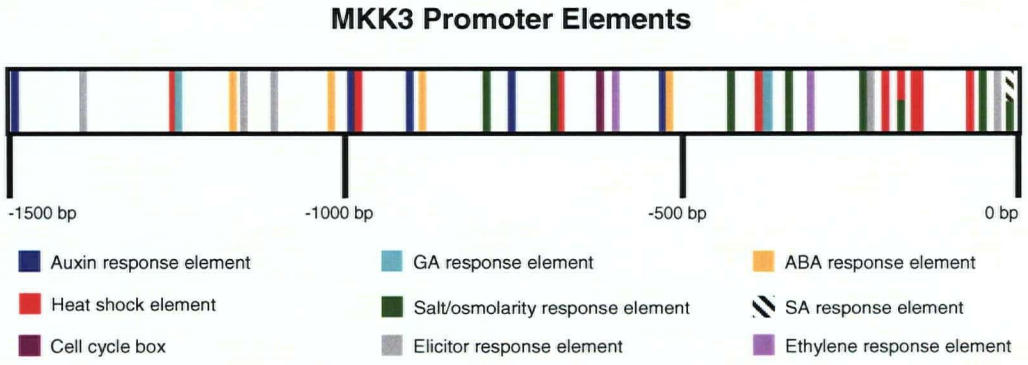

Figure 2.3: Putative CAREs encoded within *MKK3* promoter sequences. Promoter elements in the 1500 base pairs proximal to the start codon of *MKK3* were identified using PLACE Signal scan (Plant cis-acting regulatory element database; http://www.dna.affrc.go.jp/PLACE/).

In silico analysis of *MKK3* gene expression

Prior to examining *MKK3* expression in plants expressing *MKK3 promoter:GUS* reporter constructs, the expression pattern of *MKK3* was characterized *in silico* using publicly available MPSS and microarray datasets. MPSS datasets illustrated that *MKK3* is expressed consistently throughout all examined Arabidopsis tissue types, and at levels roughly similar to those of other MAPKKs (Table 2.1).

Table 2.1: Expression pattern of AtMKKs determined by massively parallel signature sequencing (MPSS). MPSS data reveal that *MKK3* (bold) is expressed at modest levels in all tissue types examined.

Locus	Kinase	Callus	Inflorescence	21-Day Leaves	21-Day Roots	Siliques 24-48 hours post fertilization
At4g26070	MKK1	46	51	72	4	42
At4g29810	MKK2	66	36	15	23	29
At5g40440	**MKK3**	**66**	**36**	**15**	**23**	**29**
At1g51660	MKK4	25	10	5	0	0
At3g21220	MKK5	51	49	32	38	18
At5g56580	MKK6	63	16	1	12	12
At1g18350	MKK7	0	0	0	0	0
At3g06320	MKK8	0	0	0	0	0
At1g73500	MKK9	121	19	38	41	35
At1g32320	MKK10	0	0	0	0	0

Examination of the public microarray datasets revealed additional characteristics of *MKK3* gene expression (Genevestigator). First, *MKK3* probes often fail to show a signal above background in these datasets, indicative of a gene with low basal levels of expression. This is consistent with both the MPSS data and with independent RT-PCR studies of *MKK3* expression (Sritubtim, 2005). In addition, *MKK3* expression was detected in all tissue types, which again was in agreement with the MPSS dataset. In these more fine-grained microarray analyses, the highest level of *MKK3* expression was reported in axillary root buds. Further mining of the public array datasets revealed that *MKK3* expression is up-regulated in response to *Pseudomonas syringae* (DC3000; ~6-fold) and *B. cinerea* infection (~2-fold), osmotic stress (~2-fold), salt (~2-fold) and senescence (~8-fold), whereas it was found to be modestly down-regulated in response to cold treatment (~1.5-fold) and cytokinin (~2-fold) exposure. Analysis of *MKK3* expression in several mutant backgrounds did not reveal any associations with previously characterized mutants (Genevestigator).

Generation of transgenic Arabidopsis plants expressing *pKK3PR:GUS*

pKK3PR:GUS transformants were initially screened on the basis of hygromycin B resistance followed by analysis of *GUS* expression in a single rosette leaf of individual T1 plants (Figure 2.4). A total of 20 T1 lines were screened for GUS activity, of which 17 showed visible staining. Among these 17 lines, patterns of expression were consistent, and most displayed low level expression typical of the images displayed in Figure 2.4 D and E. Three lines showed elevated expression patterns typical of Figure 2.4 B and C.

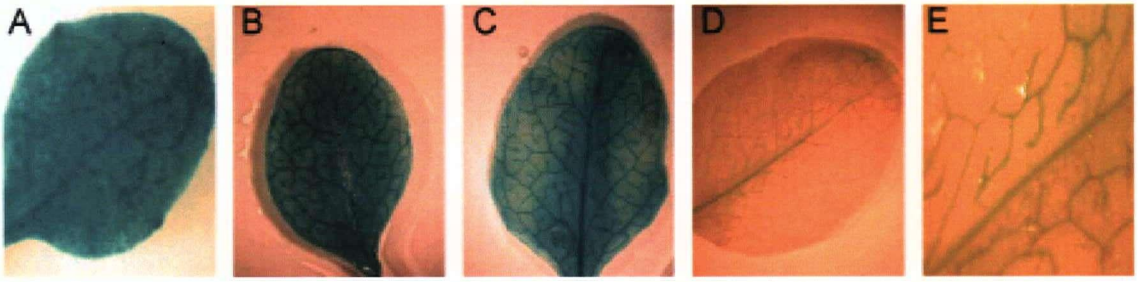

Figure 2.4: Screening for transgenic Arabidopsis plants expressing the *MKK3 promoter:GUS* reporter construct. Rosette leaves from T1 plants were harvested and analyzed for GUS activity. GUS activity controlled by *MKK3* promoter sequences varied from high to low (B-E) with all lines displaying less activity than that displayed in plants expressing the *GUS* gene under the control of the CaMV 35S promoter (A). Transgenic plants from which samples B-E were harvested were carried through for further analysis.

Two lines showing high GUS activity and two showing low GUS activity were carried through to the T2 generation, and seed collected from individual T2 plants was subjected to segregation analysis in the T3 generation to identify plants homozygous for the insertion of *MKK3 promoter:GUS* sequences. Two lines, denoted GUS1 and GUS2, displayed mid- and low-level *GUS* expression and were selected for further analysis of *MKK3* promoter-mediated gene expression. In all cases, GUS activity was consistent between these lines. Since all previous data indicated that *MKK3* is normally expressed at low levels in plant cells, high expression lines were avoided.

Analysis of MKK3 gene expression throughout plant development

To help identify possible functions of MKK3 signaling, *GUS* expression controlled by *MKK3* promoter sequences was analyzed at discrete time points throughout plant development (Figure 2.5).

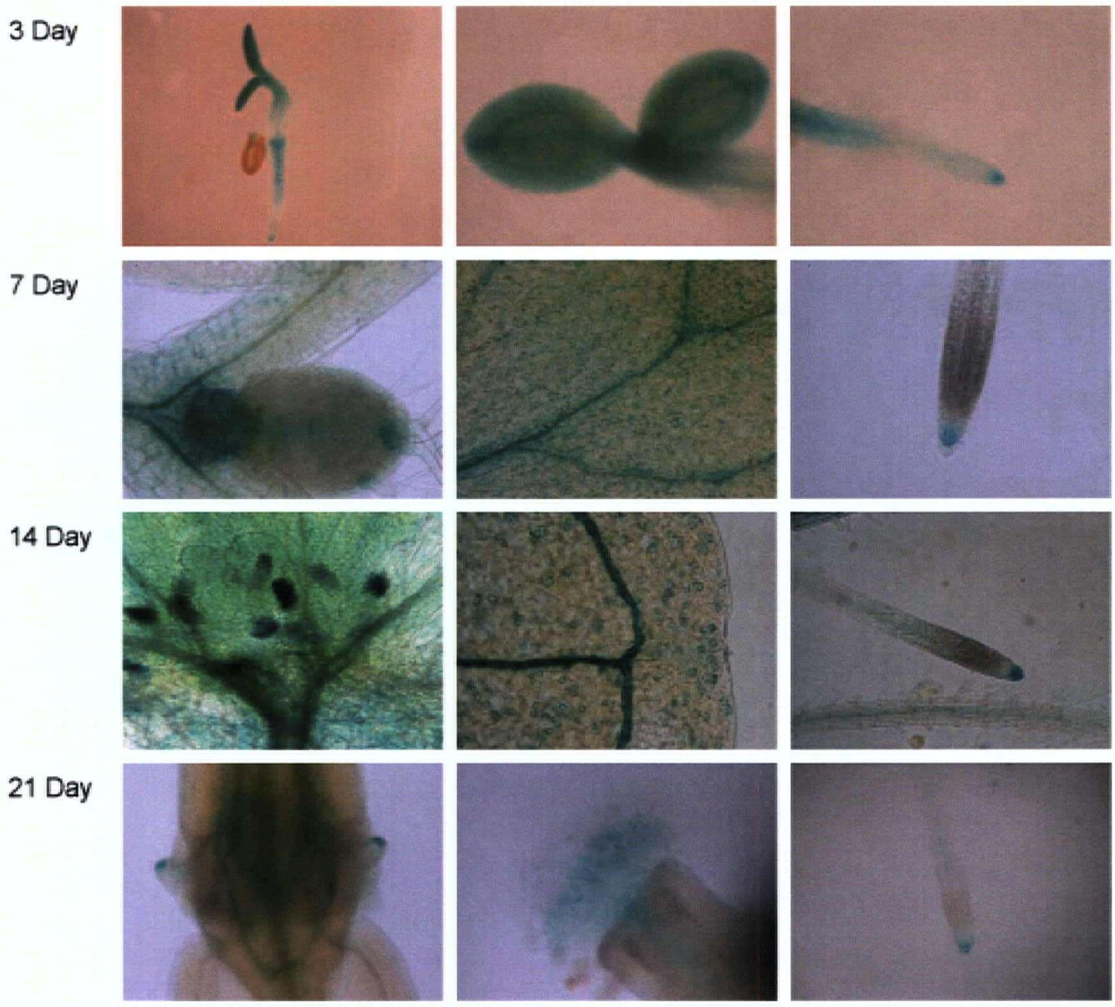

Figure 2.5: GUS activity controlled by *MKK3* promoter sequences throughout plant development. Staining patterns indicate *MKK3* promoter sequences direct expression throughout the plant at all time points analyzed. Higher-levels of expression can be seen in vasculature, guard cells, stipules, nectaries and stigma. Expression was not detected in the zone of elongation in the root tip at any time point (right panels).

Expression of *GUS* was detected in all major tissue types (stems, leaves, roots, flowers) at all time points examined. This pattern of general expression continued through to senescence, with no notable differences being detected at any time (data not shown). However, upon closer examination, specific patterns of gene expression could be seen. *MKK3* promoter-mediated GUS activity was highest in the vasculature, stipules, nectaries, guard cells and root tips (Figure 2.5). Furthermore, specific patterns of expression were seen within the root tip; expression was

always observed in the zone of cell division and zone of maturation, but no expression was detected in the zone of elongation (Figure 2.5).

The pattern of GUS activity observed in floral organs was also unique. GUS staining in the stigma was transient; expression was detected neither very early in silique development, nor after fertilization, but could be seen immediately prior to fertilization (Figure 2.6). *MKK3 promoter*-mediated *GUS* expression was also detected early in seed development, but could not be seen following synthesis of the seed coat (Figure 2.6). Proper development of seeds appeared to correlate with diminished *KK3* promoter activity whereas undeveloped seeds continued to display GUS activity even in mature siliques (Figure 2.7).

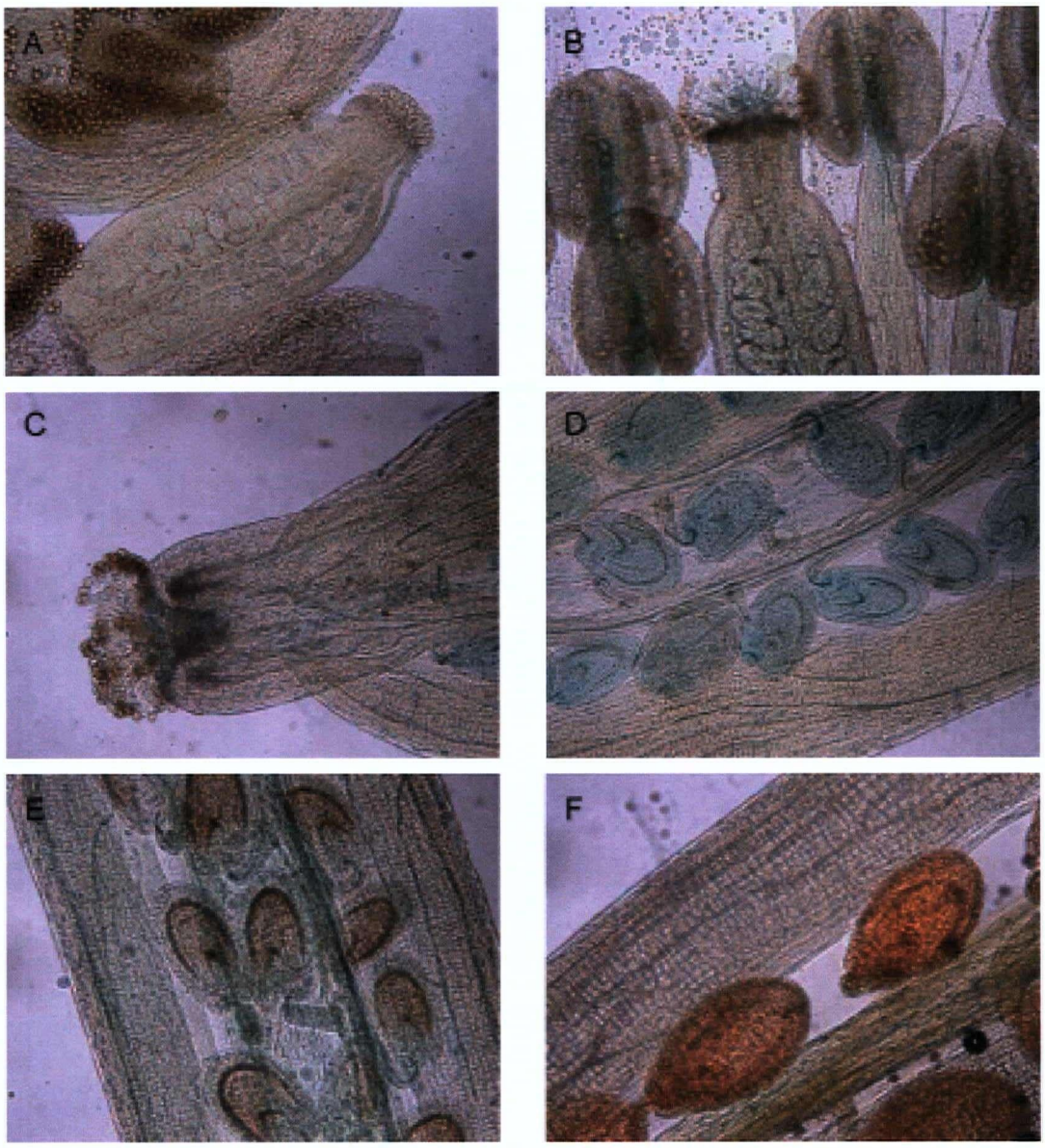

Figure 2.6. *MKK3* expression represented by GUS activity in flowers and siliques. GUS expression cannot be detected in young flowers (A), but can be observed in anthers and stigma later in floral development (B). GUS staining can be seen in floral organs as silique and seed development begins (C) and this pattern can be detected early in seed development, prior to the formation of a seed coat (D). As seed development continues, GUS staining decreases (E) until it can no longer be detected in mature seeds (F).

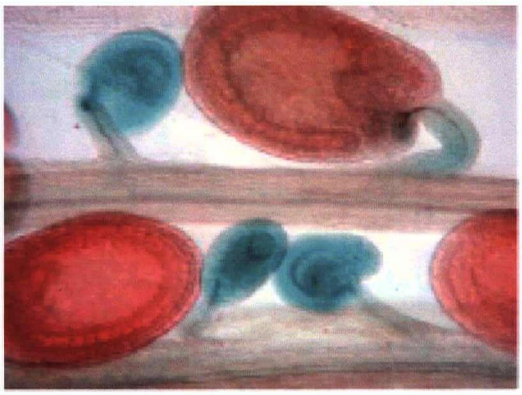

Figure 2.7. *MKK3* promoter-mediated GUS activity in undeveloped seeds. *MKK3* gene expression represented by GUS activity controlled by *MKK3* promoter sequences can be seen in seeds that fail to develop properly, even in siliques containing mature seeds.

Response of MKK3 promoter sequences to externally applied stimuli

Transgenic Arabidopsis plants expressing the *MKK3 promoter:GUS* fusion were also subjected to a panel of hormone treatments and abiotic stimuli, and resulting GUS activity changes were analyzed. Unless otherwise specified, all treatments were conducted for a period of 24 hours and GUS activity was analyzed as described (Materials and Methods).

For several of the treatments tested, *MKK3* gene expression exhibited no change (Table 2.2), suggesting that MKK3 is not transcriptionally involved in the plant response to these stimuli, at least in the "long-term" (>24 hours). However, up-regulation of *MKK3* gene expression (represented by increased GUS activity) was observed in response to a number of treatments, involving both phytohormones and abiotic stresses.

Table 2.2: Treatments that did NOT invoke changes in *MKK3* expression. Transgenic *MKK3 promoter:GUS* seedlings (14-days-old) were treated as described and GUS expression was analyzed.

Treatment	Concentration/Description
Methyl jasmonate (MeJA)	24 hour exposure to 1 μM MeJA
ACC (ethylene)	24 hour exposure to 2 μM ACC
1-naphthylphthalamic acid (NPA)	24 hour exposure to 5 μM NPA
Kinetin (cytokinin)	24 hour exposure to 0.5 μM kinetin
Gibberellin (GA)	24 hour exposure to 10 μM GA_3
Epi-brassinolide (BR)	24 hour exposure to 1 μM BR
Salicylic acid (SA)	24 hour exposure to 200 μM SA
Cold	24 hour exposure to 4°C cold treatment
Desiccation	Allow seedlings to dry for 30 minutes followed by a 4 hour recovery period on ½ MS agar plates
Wounding	Tear and pierce leaves followed by recovery periods of 30 minutes, 2 hours or 12 hours
Ozone	500 ppb for 8 hours followed by a 16 hour recovery period
Cycloheximide (CHX)	7 day exposure to 125 μg/mL CHX
Cycloheximide (CHX)	24 hour exposure to 125 μg/mL CHX
Hygromycin B	7 day exposure to 35 μg/mL hygromycin B
Sodium nitroprusside (NO donor; SNP)	24 hour exposure to 100 μM SNP
Caffeine	24 hour exposure to 2 mM caffeine
Potassium chloride	24 hour exposure to 100 mM KCl

Marked up-regulation of *MKK3* gene expression was observed in response to 100 mM NaCl (Figure 2.8). To determine if this response was dosage-dependent, plants were exposed to varying levels of NaCl (Figure 2.8). Increased GUS activity was not seen in plants exposed to NaCl concentrations <100 mM, but was observed with treatments of 100 mM NaCl or greater (Figure 2.8). Treatment with a toxic dose of 350 mM NaCl resulted in no increased *MKK3* gene expression (data not shown).

Increased expression following NaCl treatment could suggest a role for MKK3 signaling in response to either osmotic, or ionic stress, or both. To clarify this, plants were treated with

mannitol, which generates a non-ionic osmotic stress (Figure 2.9). A similar dosage-dependent activation of *MKK3* gene expression was observed with increased expression occurring following exposure to concentrations of mannitol greater than 6% (Figure 2.9).

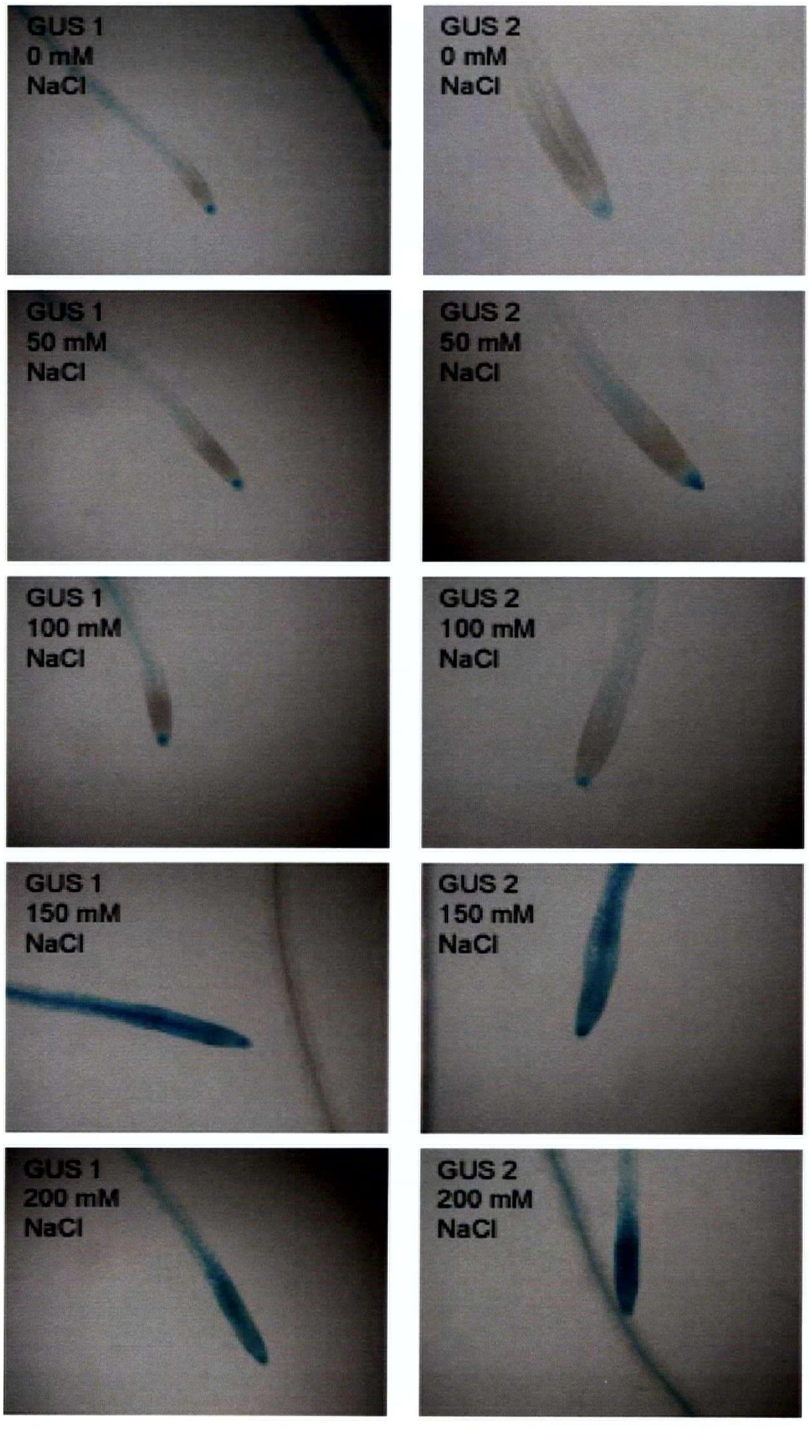

Figure 2.8: Response of *MKK3* promoter sequences to NaCl. Two independent transgenic Arabidopsis lines (GUS1 and GUS2) expressing a *MKK3 promoter:GUS* construct were treated with varying concentrations of NaCl for a period of 24 hours and the response of the *MKK3* promoter elements were analyzed via GUS activity. Increased gene expression was observed at concentrations greater than 100 mM NaCl in the root tips (primary and lateral) in both lines.

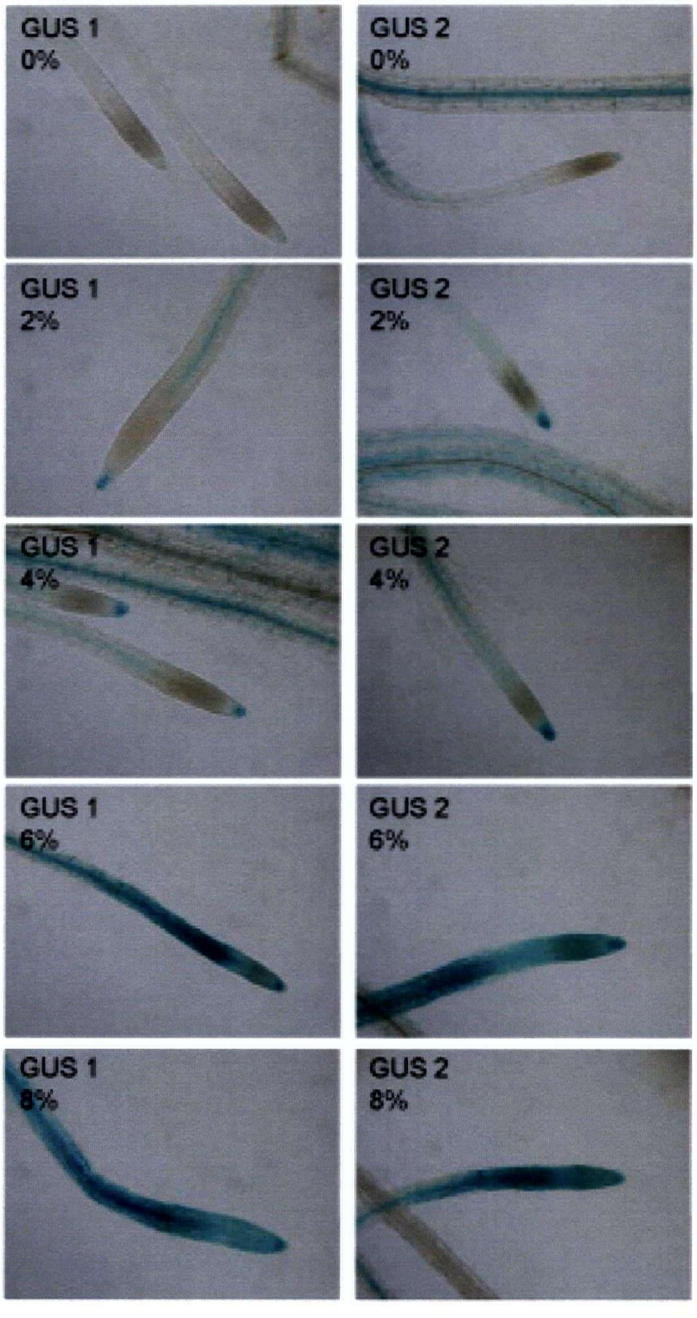

Figure 2.9: Response of *MKK3* promoter sequences to mannitol. Two independent transgenic Arabidopsis lines (GUS1 and GUS2) expressing a *MKK3 promoter:GUS* construct were treated with varying concentrations of mannitol for a period of 24 hours and the response of the *MKK3* promoter was analyzed via GUS activity. Increased gene expression was observed at concentrations greater than 4% mannitol in the root tips (primary and lateral) in both lines.

Interestingly, while *MKK3* gene expression can be detected throughout the plant, changes in *MKK3* gene expression induced by osmotic stress (NaCl; mannitol) were only observed in the

tips of primary and lateral roots. Furthermore, in some cases, the induced changes in *MKK3* expression were observed within the zone of elongation, a location in which *MKK3* gene expression is not detected in the absence of exogenous stimuli (Figure 2.9).

Treatment of *MKK3 promoter:GUS* expressing plants with 100 μM ABA also resulted in increased *MKK3* gene expression in the tips of primary and lateral roots, although not as strongly as that observed following NaCl exposure (Figure 2.10). As with NaCl and mannitol treatments, changes in *MKK3* gene expression were restricted to this region, with no changes being detected in the aerial portions of the plants.

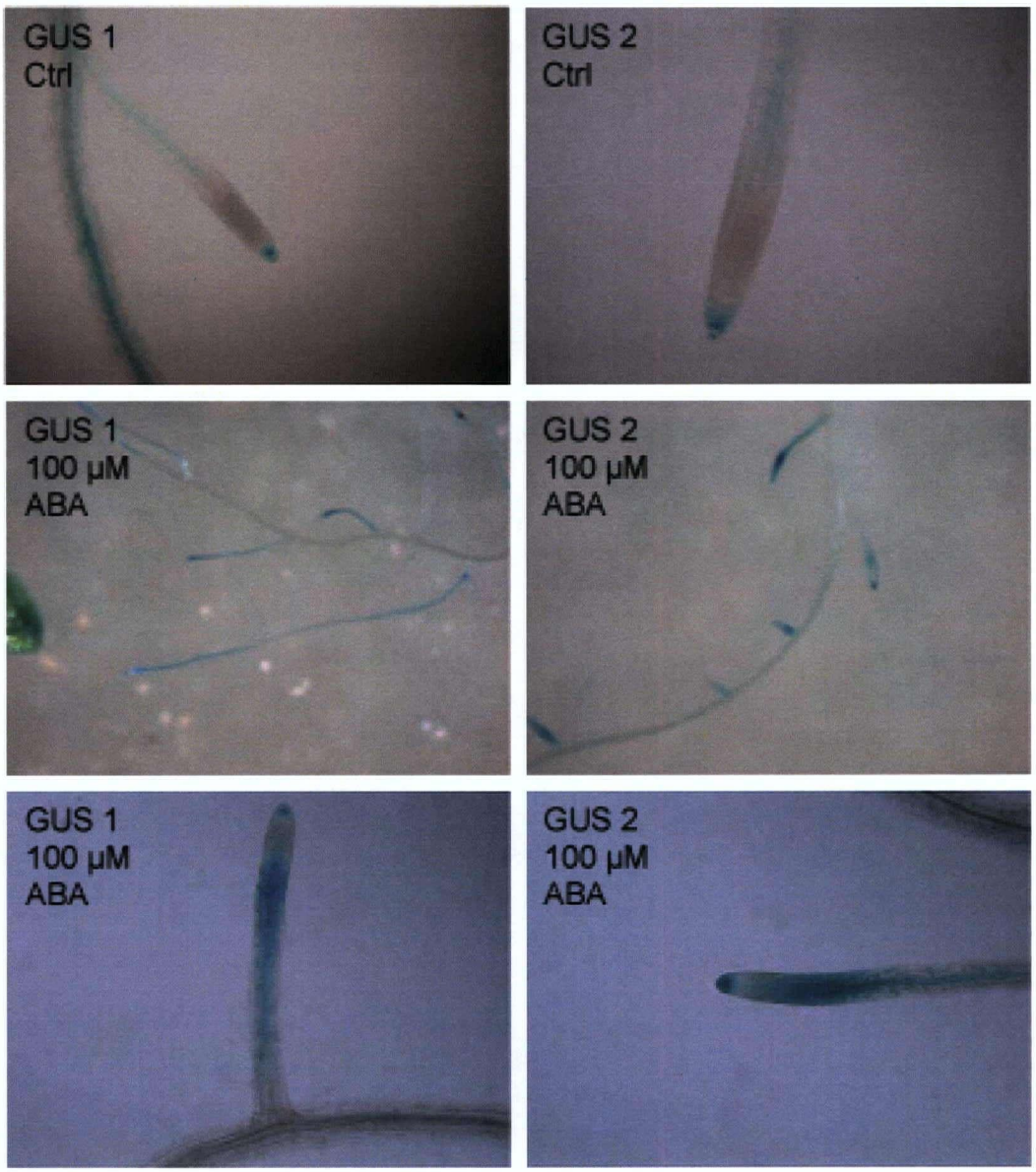

Figure 2.10: *MKK3* expression in response to treatment with ABA. 14-Day old seedlings were treated with 100 µM ABA for 24 hours followed by analysis of GUS activity. Increased *MKK3* gene expression, reflected by increased GUS activity was observed only in the tips of primary and lateral roots.

In many cases, genes that are responsive to NaCl and ABA exposure also respond to cold stress (Finkelstein and Gibson, 2002; Chinnusamy et al., 2004; West et al., 2004). However, no changes in *MKK3* gene expression were observed following exposure of plants to 4°C for a period of 24 hours but, a general up-regulation of *MKK3* gene expression was induced by a 24-

hour 37°C heat shock (Figure 2.11). Unlike other responses, up-regulation of *MKK3* gene expression in response to heat appeared to be more generalized, with increases seen throughout root tissue. However, again no changes in gene expression were observed in the aerial portions of the plant.

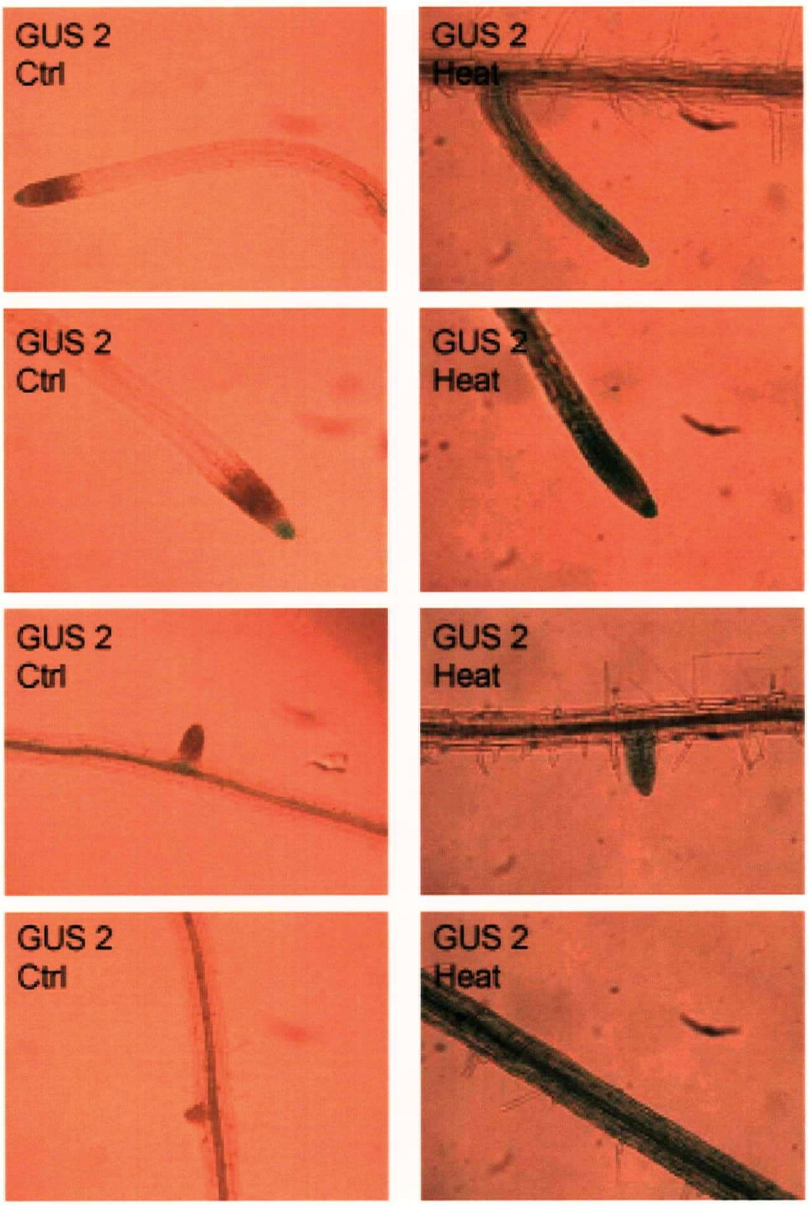

Figure 2.11: *MKK3* gene expression in response to heat shock. 14-Day-old seedlings were exposed to a heat shock of 37°C for a period of 24 hours. A general increase of *MKK3* gene expression was observed in root tissue, with a more pronounced increase observed in the tips of primary and lateral roots.

In silico analysis of *MKK3* promoter sequences had indicated the presence of putative auxin response elements (Figure 2.3; Appendix 5). Exposure of 14-day old *MKK3 promoter:GUS* plants to 1 μM IAA resulted in increased *MKK3* gene expression in the tips of primary roots, including *MKK3* gene expression within the zone of elongation (Figure 2.12). This pattern differed from other treatments that increased *MKK3* expression in root tips, since no changes in expression were observed in the tips of young lateral roots. Otherwise, as with all other cases of *MKK3* gene induction, the auxin-induced changes in gene expression were limited to roots and no response was seen in the aerial tissues.

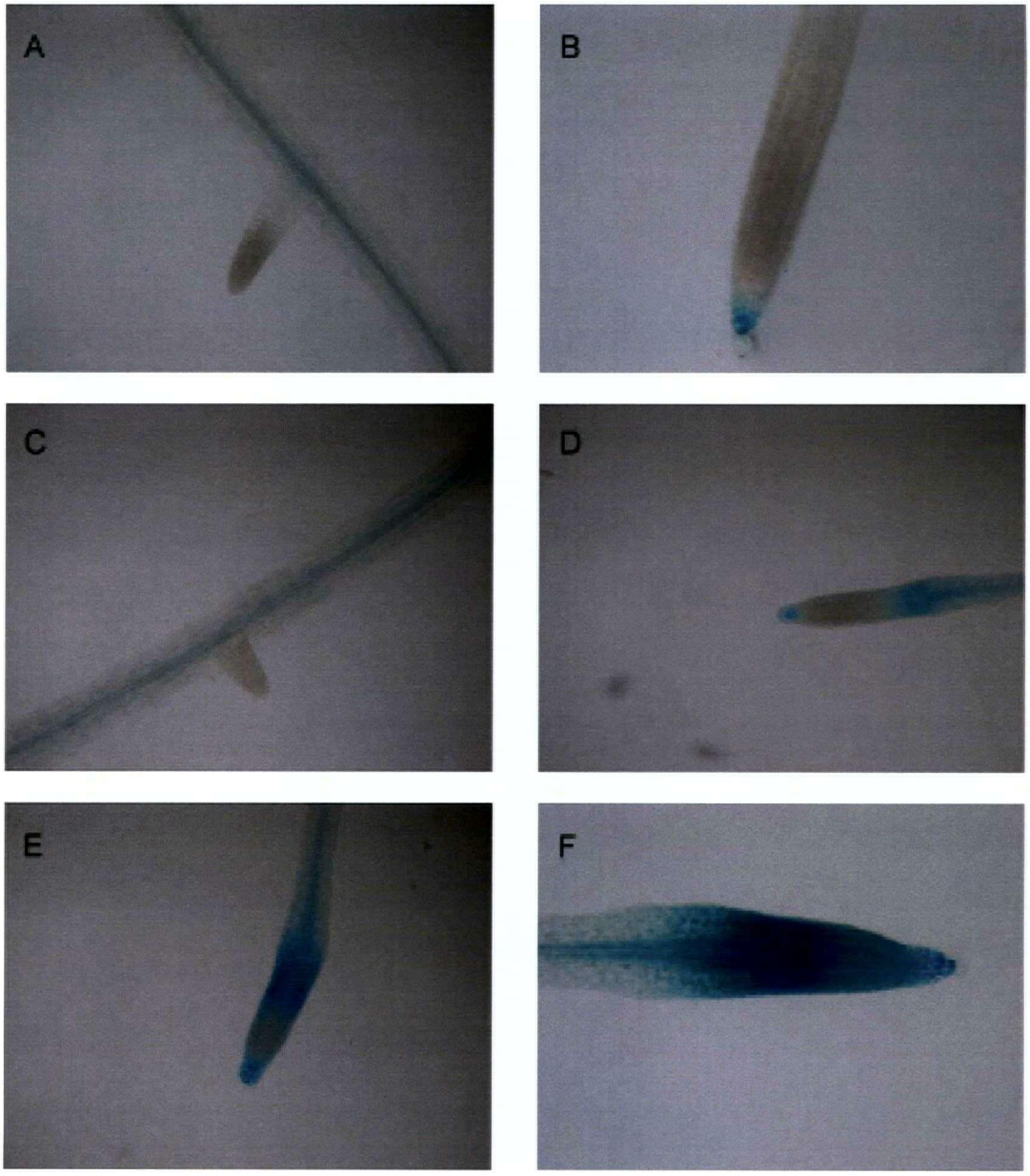

Figure 2.12: *MKK3* gene expression induced by exposure to IAA. Exposure of 14-day-old *MKK3 promoter:GUS* plants to 1 μM IAA did not result in changes in gene expression in lateral roots, but did cause increased *MKK3* expression in the tips of primary roots. (A) Untreated lateral root. (B) Untreated primary root. (C) Lateral root-bud exposed to 1 μM IAA. (D) Older lateral root (> 2-days) exposed to 1 μM IAA. (E, F) Primary root tips exposed to 1 μM IAA.

DISCUSSION

Analysis of *AtMKK3* expression by RNA-blot, microarray and RT-PCR analysis had earlier indicated that this gene is expressed in most Arabidopsis organs, including both roots and aerial parts of the plant (Ichimura et al., 1998; Sritubtim, 2005; Genevestigator). The results obtained in this study using a higher-resolution *MKK3:promoter* GUS reporter reveal additional details of *MKK3* expression. I was able to confirm that *MKK3* expression can be detected in roots, stems, leaves and floral organs but found that the expression pattern within root tissue and floral organs is very specific.

Constitutive expression of *AtMKK3* could imply that this kinase functions in the maintenance of homeostatic conditions within the plant, or that the plant maintains a constant level of MKK3 in the cell in order to allow rapid activation of signaling through MKK3 in response to activating stimuli, or both. In mammalian systems, for example, a steady-state level of inactive MEK1/2 is maintained in the cell, where it functions as a negative regulator of the MAPKs, ERK1 and ERK2, by preventing their nuclear localization. This is accomplished through the formation a heterodimeric MEK1/2-ERK1/2 cytosolic complex that only dissociates upon external activation of MEK1/2 (Kondoh et al., 2005). Constitutive expression of MAPKK genes is common and all Arabidopsis MAPKKs with the exception of MKK10 are expressed in this fashion (Hamel et al., 2006). Therefore, additional information, such as developmental response profiles and elicitor-response characteristics of individual MAPKKs are needed in order to gain insight into possible functional roles they might play in discrete cellular processes.

The *MKK3* expression pattern in floral tissues is suggestive of a function for MKK3 signaling in floral development. *MKK3* expression is transient, with expression detected in sepals, stamens and stigma, but restricted in young stamens and stigma to the period prior to fertilization

(Figures 2.5 and 2.6). Expression is also high in the nectary glands (located at the base of the silique) and in developing seeds (Figure 2.5). This pattern of gene expression in floral tissues coincides with sites of auxin production (Aloni et al., 2003; Aloni et al., 2006). The sites and timing of auxin production during floral development have been characterized using a combination of the DR5 synthetic auxin response element reporter constructs, and immunolocalisation of auxin using an auxin polyclonal antibody (Aloni et al., 2003; Aloni et al., 2006). Although the studies by Aloni et al (2003; 2006) indicate that these methods identify the sites of auxin production, they more accurately reflect only auxin accumulation. Prior to fertilization, auxin accumulates in floral tissues initially at the tips of sepals but production is then transiently observed in young petals, style and young stamens, followed by synthesis in the stigma (Aloni et al., 2006). Before synthesis appears in the stigma, auxin production occurs in the nectary glands and this latter site continues to synthesize auxin throughout development (Aloni et al., 2006). Upon fertilization, auxin is produced in increasing amounts in embryos and seeds, but this stops in late stages of seed maturation (Aloni et al., 2006). The temporal activation of auxin biosynthesis in these areas is thought to be bi-functional. Auxin signaling is associated with the promotion of development and maturation of the tissue in which it accumulates, while at the same time it appears to repress the growth and/or activity of neighboring tissues (Aloni, 2001; Taiz and Zaiger, 2002). The coincidence of *MKK3* expression and auxin biosynthesis implies that MKK3 signaling may be involved in the control of organ development, or more broadly, in controlling the response of auxin-producing cells to auxin. *MKK3* expression is also detected in both nectary glands and stipules, organs that have been reported to produce high levels of auxin (Aloni et al., 2003; Aloni et al., 2006). Stipule functions have not been characterized but it is thought that auxin produced in these cells is transported to

the shoot tip (Aloni et al., 2006). The main function of nectary glands is to secrete nectar to attract pollinators or protect against herbivores (Baum et al., 2001) and the role of auxin synthesis in these tissues is unknown.

Current hypotheses are that auxin produced in these specific floral tissues is predominantly exported to neighbouring floral tissues where it is represses local organ development (Aloni et al., 2006). Because *MKK3* expression is also detected in these auxin-source tissues it is possible that MKK3 signaling may be involved in floral development. Alternatively, *MKK3* expression may be induced following auxin biosynthesis and/or accumulation, which would suggest that MKK3 signaling is involved downstream of auxin accumulation. The *MKK3* promoter sequences are responsive to auxin (Figure 2.12). This could suggest that increased auxin levels in the tissue induce *MKK3* expression. The results of this study do not reveal if altered *MKK3* expression occurs upstream or downstream of auxin accumulation during floral development. Simultaneous detection of auxin biosynthesis/accumulation and *MKK3* gene expression using dual promoter-fluorescent protein reporters such as *DR5:CFP* and *MKK3 promoter:YFP* and auxin transport inhibitors such as NPA would help to clarify if *MKK3* expression is in response to, or leads to increased auxin production. Analysis of *MKK3* expression in auxin biosynthesis, transport and/or response mutant backgrounds would also help clarify the role of MKK3 signaling in auxin-mediated processes associated with floral development.

Although it appears that *MKK3* expression in floral tissues may be generally associated with sites of local auxin production, the *MKK3* expression pattern observed in developing seeds could be the specific result of increased ABA content during seed development. During seed development in many plant species, including Arabidopsis, two ABA concentration spikes occur. The first is seen prior to seed maturation and is maternally derived (Karssen et al., 1983; Raz et

al., 2001). The second, embryo-derived peak is approximately six-fold lower than the first in Arabidopsis (Karssen et al., 1983) and it has been suggested that it ensures maintenance of seed dormancy. However, other findings have reported that maintenance of seed dormancy is controlled by embryonic ABA produced during imbibition (Gubler et al., 2005). Nonetheless, a rigorous comparison of the timing of *MKK3* induction and hormone production in Arabidopsis seeds would help clarify whether auxin and/or ABA levels are responsible for increased *MKK3* expression during seed development.

Although several hormone- and stress-responsive CAREs can be identified in the promoter of MKK3, the *MKK3 promoter:GUS* construct was unresponsive to many of the conditions tested in the described treatment panel (Table 2.2). This could be due to a false positive prediction for a given CARE, or a failure to analyze *MKK3* expression in the appropriate physiological context. For example, while my data do not show any *MKK3* promoter response to cytokinin following 24-hour exposure, a recent microarray analysis of cytokinin-induced transcriptional responses in Arabidopsis revealed that *MKK3* expression is transiently up-regulated, with expression peaking after 6 hours of cytokinin exposure and returning to basal levels by 12 hours (Brenner et al., 2005).

Nevertheless, some of the *MKK3* CAREs appear to reflect physiological responses, since treatment with NaCl, heat, mannitol, ABA and IAA all induced *MKK3* expression in root tissue. In these instances, the lack of responsiveness of the *MKK3* promoter in aerial tissues may be due to the presence of transcriptional repressors that prevent induction of gene expression in those sites, or alternatively, the transcription factors promoting gene expression in response to these treatments in root tissues may not be expressed in aerial tissues. If *MKK3* expression in floral organs is conditioned by local auxin concentrations, it is also possible that the non-

responsiveness of *MKK3* promoter elements in aerial tissues in response to treatment with IAA could reflect a failure of the treatment method (growth of seedlings on IAA-containing medium) to supply those portions of the plant with sufficient exogenous auxin. Experiments using a different auxin delivery method, such as spraying IAA on aerial tissues, might clarify this. The pattern of *MKK3* expression in root tissue also suggests a role for MKK3 signaling in root development. Expression of the *MKK3* promoter in root tissue was concentrated in the vasculature, with the highest levels of expression observed in the meristematic region of the root tip, whereas expression could not be detected in the zone of elongation (Figure 2.5). *MKK3* expression was not detected in newly emergent lateral roots and the characteristic root-tip expression pattern was only observed in laterals two days after their emergence (Figure 2.5). As with the floral expression pattern, this pattern of *MKK3* expression coincides with known sites of auxin accumulation in roots (Benkova et al., 2003; Ljung et al., 2005). The accumulation of auxin in these tissues is thought to play a central role in the developmental programming associated with root tissue patterning (Blilou et al., 2005; Ljung et al., 2005), and the correlation of auxin accumulation and *MKK3* expression is suggestive of a function for MKK3 in this process. Recently, Birnbaum et al (2003) produced a global expression map of the Arabidopsis root in which they characterized gene expression profiles of 15 discrete zones in the root, representing different cell types and developmental stages (Birnbaum et al., 2003). Auxin-inducible genes were over-represented among those being expressed in the zone of differentiation, another region of the root that shows significant *MKK3* expression (Birnbaum et al., 2003). Consistent with this, *MKK3* expression in the root appears to be controlled at least in part by auxin, based on the dramatic increase in *MKK3* promoter expression observed in primary root tips following IAA treatment (Figure 2.12); this pattern can also be observed in the publicly

available auxin-response microarray datasets (Genevestigator). It would be interesting to analyze *MKK3* expression in auxin-deficient or transport mutants that display root pattern defects, such as *monopteros, axr3* or *pin4* (Berleth and Sachs, 2001).

Since one of the classic physiological responses to auxin application is increased lateral root formation, the failure to detect *MKK3* expression in lateral root buds is somewhat surprising (Casimiro et al., 2003). *MKK3* expression was only observed in the tips of primary roots, and in the tips of lateral roots greater than two days old (Figure 2.12). This suggests that MKK3 signaling may be involved in aspects of the root auxin response that are not essential for lateral root initiation. Interestingly, a different MAPKK, AtMKK6 has recently been found to be associated with lateral root initiation in Arabidopsis (Sritubtim, 2005).

Exposure of *MKK3 promoter:GUS* plants to NaCl, mannitol or ABA resulted in dramatic increases in *MKK3* expression in root tips. Unlike the response to auxin, these treatments induced increased *MKK3* expression in all root tips, including newly emergent lateral roots. Furthermore, *MKK3* expression could also be seen in the zone of elongation following these treatments, a region usually devoid of *MKK3* promoter activity. Plants typically respond to ABA, NaCl and osmotic stress by ceasing root growth (Finkelstein et al., 2002; West et al., 2004). Increased auxin can induce a similar response, since primary root growth was inhibited following addition of exogenous auxin to Arabidopsis plants grown in tissue culture medium (Lincoln et al., 1990). Perhaps the increased *MKK3* expression in areas of diminished root growth reflects some role for MKK3 in controlling root growth. Interestingly, the response of the *MKK3* promoter to NaCl was dosage dependent. Increased *MKK3* expression was detected at NaCl concentrations greater than 100 mM but below 350 mM. WT plants exposed to a NaCl gradient only display growth inhibition between NaCl concentrations of >50 mM and 220 mM

(Lehle et al., 1992). Exposure to NaCl concentration less than this result in normal root growth, while exposure to NaCl at concentrations above 220 mM result in plant death (Lehle et al., 1992). Thus the NaCl induced *MKK3* expression pattern correlates with growth inhibiting concentrations and could reflect a role for MKK3 signaling in salt tolerance. A similar dose-response pattern was observed for the response of the *MKK3* promoter to mannitol, which generates a non-ionic osmotic stress (Werner and Finkelstein, 1995); increased *MKK3* promoter activity was only detected following exposure to levels of mannitol that result in significant root growth inhibition (Arabidopsis Ganlet Project; http://thale.biol.wwu.edu/index.html).

MKK3 expression is also induced by extended heat shock (24 hours at 37°C). Unlike other *MKK3*-inducing treatments, heat stress resulted in a general up-regulation of *MKK3* expression in all regions of root tissue (Figure 2.11). It is known that there is significant overlap between the sets of genes induced by heat, drought and osmotic stress (Zhu, 2002; Rizhsky et al., 2004). In my experiments, the heat-exposed plants were cultivated and heat-treated on sealed plates containing gel-solidified aqueous medium, so they should not have been subject to dehydration. I therefore conclude that this *MKK3* response is likely to be heat-specific. Because the plants were exposed to a relatively long heat shock, I assume that the *MKK3* expression changes observed are more likely associated with acquired heat tolerance than with basal, acute thermotolerance. It was recently reported that acquired heat tolerance and basal thermotolerance are distinct phenomena, and that each involves multiple signaling pathways in Arabidopsis, with ethylene, salicylate and antioxidant metabolism being linked to basal thermotolerance, while ABA was critical to acquired thermotolerance (Larkindale et al., 2005). During acquisition of increased heat tolerance, plants are thought to accumulate ABA; thus, ABA biosynthetic and response mutants are hypersensitive to heat stress, while pre-treatment of plants with ABA, or

over-accumulation of ABA in certain mutant backgrounds, confers enhanced heat tolerance (Larkindale et al., 2005). Since *MKK3* expression also responds to increased ABA, analysis of *MKK3* expression in both heat-tolerant and heat-sensitive ABA-deficient mutants would help establish whether *MKK3* induction by heat stress is directly associated with the heat treatment, or is a consequence of heat-induced ABA accumulation. Analysis of *MKK3* expression during acute heat-stress would also help clarify this, although acute heat tolerance is supposedly associated with ethylene and salicylate signaling (Larkindale et al., 2005), and neither of these phytohormones was found to affect *MKK3* gene expression in my survey. This suggests that *MKK3* signaling is more likely involved in processes associated with acquired heat tolerance, which could include decreased root growth.

Analysis of publicly available microarray datasets indicated that *MKK3* expression can be induced by osmotic stress, salt and ABA exposure, all of which correspond with the results of my promoter:reporter study. On the other hand, other factors reported in the database to influence *MKK3* promoter activity either could not be confirmed, or were not examined (Table 2.3). First, *MKK3* expression was apparently up-regulated during senescence in Arabidopsis, according to Genevestigator analysis, but no such senescence-related expression was observed in *MKK3 promoter:GUS* plants. However, the model of senescence used in the reported microarray experiment involved nutrient-deprived 14-day old suspension culture cells, rather than developmental senescence in intact plants. In a recent transcriptional profiling experiment of senescing Arabidopsis leaves, Lin and Wu (2004) also did not detect any up-regulation of *MKK3,* suggesting that MKK3 is not involved in developmental senescence (Lin and Wu, 2004).

Table 2.3: MKK3 expression profiles detected using Genevestigator. *MKK3* expression patterns reported in publicly available datasets were examined using Genevestigator (https://www.genevestigator.ethz.ch/).

Treatment/condition	Approximate Fold-Change
Pseudomonas syringae DC3000	+6
Syringolin	+4
B. cinerea	+2
Osmotic stress	+2
Salt exposure	+2
Senescence	+8
Cyokinin	-2
Cold stress	-1.5

A recent comparison of global expression profiles associated with three different patterns of senescence also concluded that, while many genes responded similarly during cell death-associated senescence, nutrient deprivation-associated senescence and developmental senescence in Arabidopsis, there were also many genes whose expression changes were uniquely associated with each of those physiological processes (Buchanan-Wollaston et al., 2005). However, there was no evidence suggesting that *MKK3* expression is associated with any of these processes. *MKK3* expression was also reported in Genevestigator to be induced by interaction of Arabidopsis plants with the phytopathogens *P. syringae* and *B. cinerea*. *MKK3* expression in response to pathogens was not examined in my study, but since multiple pathogens and the bacterial elicitor, syringolin A, which is derived from *P. syringae* (Waspi et al., 2001), appear to induce *MKK3* expression, the role of MKK3 signaling in response to pathogens clearly should be examined further.

An *MKK3* down-regulation response to cold treatment and to cytokinin exposure was also reported in the public databases (Table 3.3). My analysis of *MKK3:promoter GUS* plants did not detect such a response, but since the expression differentials reported in the array database were small (1.5- and 2-fold) it is possible that the persistence of the GUS reporter in these plants may

have hindered my ability to detect small decreases in *MKK3* promoter activity. Promoter-GUS reporter systems also will not reveal decreased gene expression that results from mRNA degradation by regulatory RNAs such as miRNAs, and it has recently become clear that plant gene expression is often controlled at this level, especially in the case of auxin-associated genes (Chen, 2005; Guo et al., 2005; Hardtke, 2006). The findmiRNA miRNA prediction algorithm (http://sundarlab.ucdavis.edu/mirna/) predicts three possible miRNAs capable of targeting *MKK3* transcripts, suggesting that *MKK3* expression may be controlled by this mechanism. One of these predicted putative miRNA species (5' UAUCUCUGUAACCUCCUCG 3') displayed significant homology to AtMPK7 suggesting that abundance of both of these genes could be controlled by this same miRNA. In addition, both this miRNA and another predicted miRNA (GUCUUUCUAGGUCUGGGAG) displayed significant homology to AtMPK8.

CONCLUSIONS

The constitutive expression of *MKK3* detected in this study suggests that MKK3 might function in maintenance of homeostatic conditions within the plant. MKK3 would seem to have the potential to form protein-protein regulatory complexes, based on the presence of a C-terminal NTF2 domain in the protein. Although the molecular function of the NTF2 domain in MKK3 is currently unknown, examples of other NTF2 domain-containing proteins from other organisms, such as TAP and Mex67, illustrate that this domain is involved in protein:protein interactions (Quimby et al., 2000; Stewart, 2000; Chaillan-Huntington et al., 2001; Thakurta et al., 2004). The responsiveness of *MKK3* promoter sequences to the stress-related hormone, ABA, as well as to salt, osmotic and heat stress treatments suggests, possible additional roles for MKK3 signaling in Arabidopsis stress responses. Finally, correlation of *MKK3* expression patterns with sites of auxin biosynthesis and accumulation may indicate a role for MKK3 signaling in development.

One common factor in all of these scenarios is the association of increased *MKK3* expression with induced growth arrest, since salt, heat, osmotic stress, ABA and auxin accumulation all result in some degree of growth inhibition. Therefore, the picture emerging from these gene expression analyses is one in which MKK3 may somehow participate in the global negative regulation of growth in Arabidopsis tissues. The nature of this hypothetical role, and whether it might be essential or redundant, cannot be addressed by simply monitoring expression of MKK3 gene, but requires additional 'reverse genetics' approaches.

CHAPTER 3: Characterization of *MKK3* loss-of-function plants

INTRODUCTION

The apparent convergence of MAPK signaling at the MAPKK level implies that specific MAPKKs are likely to be substrates of multiple MAPKKKs, and that each MAPKK may be capable of phosphorylating multiple MAPKs. Thus, study of signaling at the MAPKK level is of particular interest since it is likely that signal integration between multiple signal transduction pathways occurs via these kinases.

Phylogenetic analysis of the MAPKK gene family in Arabidopsis has placed these kinases into four groups, A-D. Group B MAPKKs are unique in their possession of a 'nuclear transport factor 2' (NTF2) domain in addition to the characteristic dual-specificity Ser/Thr-Tyr kinase domain (Ichimura et al., 1998; Ichimura et al., 2002). Group B MAPKKs such as AtMKK3 appear to be evolutionarily conserved, with homologues identified in both close and distant relatives, including tobacco, rice, poplar, *Selaginella* and *Chlamydomonas* (Shibata et al., 1995; Ichimura et al., 1998; Ichimura et al., 2002; Hamel et al., 2006). In Arabidopsis, poplar and rice, plants for which the genomes have been fully sequenced, group B MAPKKs form a single member clade, suggesting that this is the case in most, if not all, higher plant species. In *Chlamydomonas,* the putative MKK3 orthologue is the only MAPKK encoded in the genome (Hamel et al., 2006), suggesting that the group B/MKK3 class of plant MAPKKs may represent the archetype. From a functional genetics perspective, this singularity could also possibly offer an advantage, in that other MAPKKs may be less likely to act redundantly in group B MAPKK loss-of-function plants.

The only functional data previously available for AtMKK3 showed that the gene is expressed in all major plant organs (Ichimura et al., 1998), while the results presented in Chapter 2 suggest a possible role for MKK3 signaling in development and/or stress induced phytohormone signaling, particularly in the inhibition of root growth in response to stresses and hormones. Phenotypic characterization of MKK3-loss-of-function mutants could highlight additional aspects of the biological functions of MKK3 signaling modules.

Unlike some other eukaryotic organisms, efficient site-directed manipulation of plant genomes by homologous recombination has not yet been achieved (Schuermann et al., 2005). Nonetheless, several tools are available for generating loss-of-function mutants in Arabidopsis, including T-DNA insertional mutants and RNA interference. Large scale collections of T-DNA insertional mutants have been generated and made available to the community through the Arabidopsis Biological Resource Centre (ABRC; www.arabidopsis.org). Currently several T-DNA insertional mutant collections are now available through ABRC, including the original Salk Institute-generated collection (Alonso et al., 2003), SAIL lines generated by Syngenta (Sessions et al., 2002), T-DNA lines provided by the Arabidopsis Functional Genomics Consortium (AFGC; Madison, WI, USA), and collections generated in other functional genomics projects in Japan and Germany. Other approaches are also used to generate targeted loss-of-function mutants, the most frequent of which is gene-silencing by anti-sense suppression, which is now known to be related to RNA-interference (RNAi; Watson et al., 2005).

To gain insight into the biological function of MKK3 signaling, I wished to examine the phenotypic consequences of diminished signaling through MKK3 in MKK3 loss-of-function plants. For that purpose, I examined the one available MKK3 T-DNA insertion mutant and also created a series of transgenic Arabidopsis plants in which *MKK3* gene expression was targeted

for reduction via RNAi-mediated gene silencing. Because constitutive expression of an *MKK3-RNAi* construct could potentially interfere with plant development, the RNAi construct was placed under the control of a dexamethasone-inducible promoter system in the *pTA7002* vector system (Aoyama and Chua, 1997).

MAPK signaling modules are known to often activate transcription factors, thereby invoking large-scale changes in gene expression (Yang et al., 2003; Feilner et al., 2005; Menke et al., 2005). Since these changes in gene activity can be expected to reflect the biological context in which MKK3 signaling operates, it would clearly be informative to identify genes whose expression changes in response to manipulation of MKK3 activity. I therefore examined the molecular phenotype associated with insertion of T-DNA into the MKK3 locus, through full transcriptome microarray analysis using a 70-mer "long oligo" array (Douglas and Ehlting, 2005; Ehlting et al., 2005; Ro et al., 2005). Genes associated with MKK3 signaling were identified by comparing the transcript profiles of both untreated 10-day-old tissue culture-cultivated SALK 051970 MKK3 T-DNA insertion seedlings, and untreated 20-day-old rosette leaves harvested from SALK 051970 plants, with correspondingly handled WT plants.

Since possession of an NTF2 domain is a major feature of the MKK3 gene, I also wanted to try to gain further insight into its role. For that purpose, gene expression profiles were examined in independent transgenic Arabidopsis lines over-expressing either a full-length version of *MKK3*, or a truncated, *MKK3ΔNTF2* variant, each expressed under the control of the CaMV 35S promoter in the MKK3 T-DNA insertional background.

MATERIALS AND METHODS

Plant Lines

Arabidopsis thaliana Col-0 was defined as wild-type (WT) for all assays in this work. All Arabidopsis plants were cultivated in Redi-earth® (Sun Gro Horticulture, Vancouver, BC, Canada) and grown at 21°C under a 16 hour: 8 hour light: dark cycle.

SALK 051970/MKK3 T-DNA Insertional Mutant Line

The MKK3 T-DNA insertion line, SALK 051970, was identified using the SIGnAL "T-DNA Express" Arabidopsis Gene Mapping Tool (http://signal.salk.edu/cgi-bin/tdnaexpress). This line contains a T-DNA insert in exon 7 of the gene encoding MKK3, which lies at the midpoint between the dual-specificity kinase domain and the NTF2 domain (Figure 2.2). T3 segregating seeds of SALK 051970 were obtained from ABRC (www.arabidopsis.org) and propagated to identify lines homozygous for the T-DNA insertion at this locus. Plants homozygous for the insertion were screened using standard PCR conditions (Appendix 1) with primers KK3ScreenF (ATG CTC GAC CAA CAG CTG ACC) and KK3ScreenR (GAG AAC AAA CGT TTT CTC ATG TGT G) whose targets flank the T-DNA insertion. Verification that the T-DNA insertion eliminated full-length *MKK3* transcripts was conducted via RT-PCR analyses using primers MKK3FL-F (ATG GCG GCA TTA GAG GAG CTA) and MKK3-3'UTR-R (ATA GTA CAG TAG AGA ACA AAC G). WT plants, and plants heterozygous for the insertion, both produce a 1604 base pair amplicon using these primers whereas lines homozygous for the insertion yield no amplicon.

35S:MKK3 and 35S:MKK3ΔNTF2 over-expression lines

The MKK3 T-DNA insertional mutant line was complemented via two methods. A transgenic line over-expressing full-length *MKK3* under the control of the CaMV 35S promoter was generated by placing the complete *MKK3* open reading frame (ORF) downstream of the CaMV 35S promoter sequences in the binary vector *pGL-CAMBIA-35S*. Full-length *MKK3*, including a portion of the 3'UTR was amplified by PCR from cDNA template derived from 21-day old rosette leaves using the PCR primer pair, MKK3FL-F (see above) and MKK3-3'UTR-R (ATA GTA CAG TAG AGA ACA AAC G) and Platinum-Taq HIFI (Invitrogen, Burlington, ON, Canada). The amplified ORF was inserted into the *pYES2.1* vector (Invitrogen, Burlington, ON, Canada) by TOPO-TA mediated cloning, generating the plasmid *pYES-KK3-3HA*. Following validation of this clone by DNA sequence analysis (NAPS, UBC), *MKK3* sequences were PCR-amplified using MKK3IFF (AGG ACC TCG AGA ATT TTA TCA TGG CGG CAT TGG AGG AGC) and MKK3-3'UTR-R (ATT TGC GGA CTC TAG ATG CCG CCC TCT AGA AAC TCA ATG) primers and subcloned into the binary vector *pGL-CAMBIA-35S* using the BD-In fusion recombination kit (BD Biosciences), thereby generating the plasmid *p35S-MKK3* (Figure 3.1).

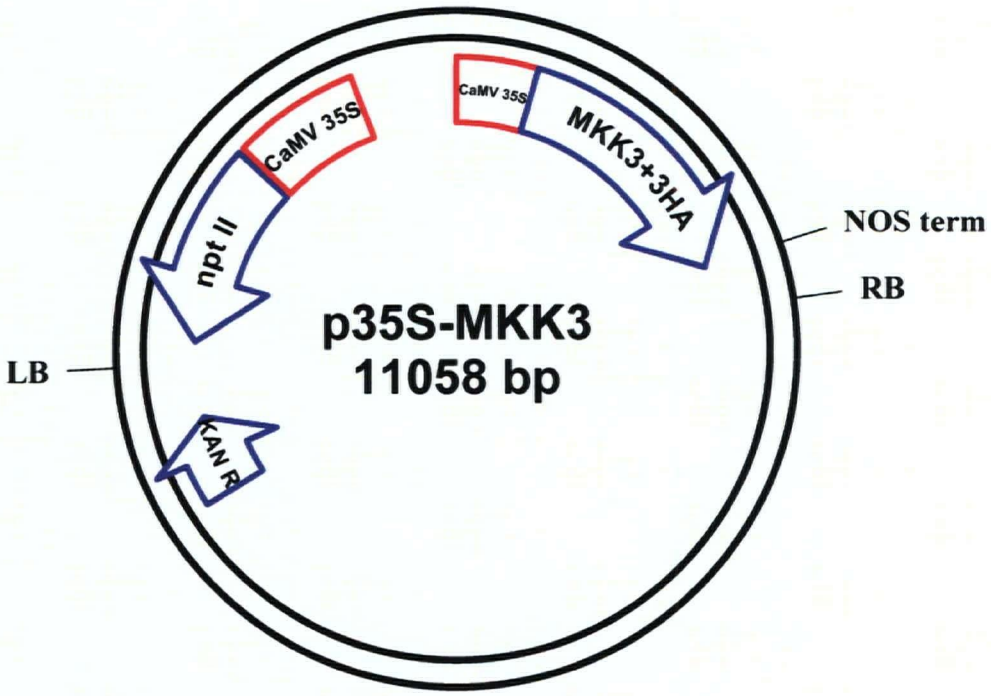

Figure 3.1. ***pGL-35S-KK3.*** The full length cDNA encoding MKK3, with addition of 3' His_6 and triple hemagglutinin (HA) epitope tags, was fused downstream of the CaMV 35S promoter sequences contained within the binary vector *pGL-CAMBIA-35S.*

The SALK 051970 MKK3 T-DNA insertion was also complemented by over-expression of a variant of *MKK3* from which the NTF2 domain had been removed (*MKK3ΔNTF2*). The *NTF2* domain was eliminated from the full length *MKK3* sequences contained within *pYES-KK3-3HA* by taking advantage of the ability of yeast to undergo homologous recombination (Figure 3.2).

Figure 3.2. Synthesis of the *MKK3ΔNTF2* variant. The full-length *MKK3-3HA*-containing plasmid *pYES-KK3-3HA* was linearized by digestion with *Hind*III and yeast strain YPH499 was co-transformed with the linearized plasmid and a linear fragment of DNA containing elements required for deletion of the NTF2 domain encoding sequences by homologous recombination (amplified product for NTF2 deletion). A double recombination event between the linearized plasmid and PCR product in the 5' (purple) and 3' (italics) flanking regions generated an *MKK3ΔNTF2* variant. Positive recombinants were screened by PCR analysis and sequence integrity of the recombined product was verified by DNA sequence analysis.

The sequences 3' of the NTF2 domain through to the triple HA tag were amplified by PCR using the primers KK3ΔNTF2-F (AGA GTA GAT TTG GCG ACT TTT GTT CAA AGC ATC TTT GAT CCA ACT TTT GTT GTT GAA TCT GGT GAT CTT) and KK3ΔNTF2-R (ATC GTA TGG GTA ATC TAA GTT TGT). The forward primer contains 45 base pairs of sequence that are homologous to sequences immediately 5' of the NTF2 domain (Figure 3.2 purple). When the resultant amplicon was co-transformed into yeast with linearized *pYES-KK3-3HA* homologous recombination occurred between the sequences 5' of the NTF2 domain in addition to a second recombination occurring between the sequences downstream of the NTF2 domain. This resulted in the synthesis of a recombined plasmid in which the NTF2 domain encoding sequences have been removed, while preserving sequences encoding the C-terminus of MKK3 and the downstream triple HA and poly-histidine epitope tags.

Insertion of the *MKK3ΔNTF2* sequences into the binary vector *pGL-CAMBIA-35S* was carried out as with the full-length MKK3 clone, using the BD-In fusion recombination kit (BD Biosciences, Mississauga, ON, Canada) and the primers, MKK3IFF and MKK3-3'UTR-R, to generate the plasmid *p35S-MKK3dNTF2* (Figure 3.3).

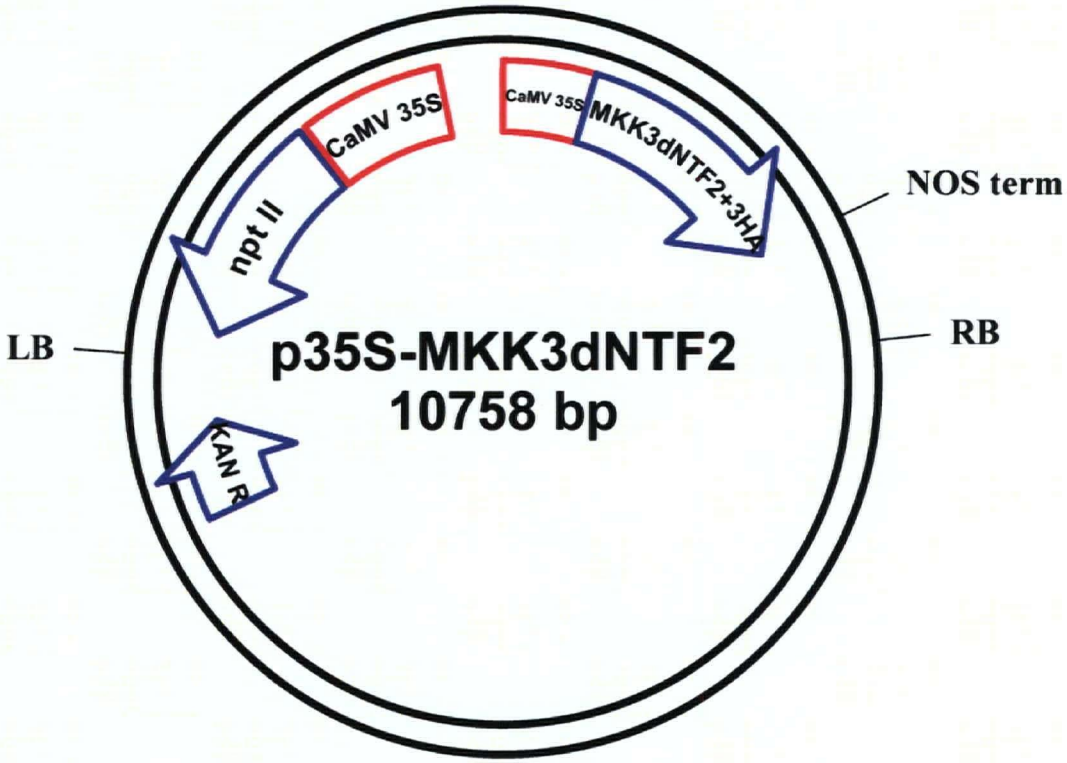

Figure 3.3. *p35S-MKK3dNTF2* binary vector used to transform the SALK 051970 T-DNA insertion line. *MKK3ΔNTF2-3HA* sequences were cloned downstream of the CaMV 35S promoter in the binary vector *pGL-CAMBIA-35S* to enable constitutive expression in host plant cells.

The binary vectors carrying the *35S:MKK3* variants were independently introduced into competent *Agrobacterium tumefaciens* EHA105 cells, which were used to transform the SALK 051970 MKK3 T-DNA insertion line via the floral dip method (Appendix 1, Materials and Methods). T1 transformants were selected on the basis of resistance to hygromycin B. Plants

that were resistant to hygromycin B were transferred to soil and expression of the transgene in 35 independent transgenic plants derived from each transformation was assessed by RT-PCR (data not shown). Two plant lines displaying the highest level of transgene expression for each of the *MKK3-3HA* and *MKK3ΔNTF2-3HA* transgenes were allowed to grow until seed set. T2 seeds heterozygous for the transgene insertion were germinated in the presence of hygromycin B and resistant plants were transferred to soil and cultivated until seed set. To identify plants homozygous for the insertion, T3 seeds collected from independent T2 plants were germinated in the presence of hygromycin B and scored for hygromycin B resistance. Seed stocks displaying 100% germination in the presence of hygromycin B over two independent trials were deemed to be homozygous for the transgene insertion.

MKK3 RNA-interference (RNAi) plants

To provide an alternate method of eliminating MKK3 signaling from the plant, a RNA-interference (RNAi) construct specific to the N-terminus of MKK3 was synthesized. This construct was placed under the control of a dexamethasone-inducible promoter system (Aoyama and Chua, 1997) in order to allow temporal induction of *MKK3* silencing. This dexamethasone-inducible promoter system requires constitutive expression of an inducible transcription factor, referred to as GVG which in this instance in controlled by a dual 35S promoter system (Aoyama and Chua, 1997). The GVG transcription factor is a chimeric protein in which the ligand binding domain of a rat glucocorticoid receptor (G), the herpes virus VP16 protein (V) and the N-terminus of the yeast Gal4 transcription factor (G) are fused in sequence. In the absence of dexamethasone, the GVG transcription factor remains cytosolic, sequestered there by the heat-shock protein, HSP90. Upon binding to dexamethasone, HSP90 is displaced and the GVG moves to the nucleus where it binds to a hexameric repeat of the GAL4 upstream activating

element. Since the latter is located upstream of the gene of interest, activation of the GAL4 promoter results in high levels of induced gene expression (Aoyama and Chua, 1997). To maximize the potential that the RNAi construct would be specific to MKK3 sequences and not inadvertently silence other Arabidopsis MAPKKs, a 211 base pair sequence extending from 129 base pairs downstream of the *MKK3* translation initiation codon to position 340 was selected as the target for the RNAi construct. This particular sequence lies within the most unique region of MKK3 which is only 53% identical to the closest MAPKK, MKK2 (Table 3.1). Additionally, no strings of sequence identify greater than 10 base pairs in length were observed between this region and any MAPKK.

Table 3.1. Percent identity of *MKK3 RNAi* target sequence and genes encoding the remaining Arabidopsis MAPKKs

MAPKK	% Identity
MKK1	43
MKK2	53
MKK4	49
MKK5	52
MKK6	50
MKK7	45
MKK8	45
MKK9	45
MKK10	51

The sense strand portion of the construct was generated by PCR amplification using Platinum Taq HIFI (Invitrogen, Burlington, ON, Canada) and the PCR primers KK3RiSF (G CCT CGA GCT TAG TAG ATC ATA TGG AGT) and KK3RiSR (AGA GAA TTC CTA TGA GCT GCA AAA ACT ACT TAC CTC TCT TCT TCA ACG CTA AAA TTC TAT GAT TAG) and cloned via TOPO-TA mediated cloning into the cloning vector, *pCR2.1* (Invitrogen, Burlington, ON, Canada). The KK3RiSR primer includes sequences encoding a short synthetic intron identical to that used by Samuel et al (2002) to silence *WIPK* expression. This sequence was designed to be

spliced out following transcription of the RNAi construct, resulting in the formation of the appropriate double-stranded RNA species required to trigger RNAi-based gene silencing. The anti-sense portion of the *MKK3 RNAi* construct was created through amplification of the target region by PCR using Platinum Taq HIFI (Invitrogen, Burlington, ON, Canada) and the PCR primers KK3RiASF (C GAC TAG TCT TAG TAG ATC ATA TGG AGT) and KK3RiASR (AG GAA TTC TCT TCT TCA ACG CTA AAA TTC TAT GAT TAG), followed by insertion of the amplified product into *pCR2.1* (Invitrogen, Burlington, ON, Canada) by TOPO-TA-mediated cloning. Following sequence verification of each of these clones, the sense and anti-sense fragments were excised from the corresponding plasmids by double restriction digestion with *XhoI/EcoRI* and *EcoRI/SpeI* enzyme pairs, respectively. Concurrently, the binary vector *pTA7002* was linearized by digestion with *XhoI* and *SpeI*, and dephosphorylated using Antarctic shrimp alkaline phosphatase (New England Biolabs, Pickering, ON, Canada). All three fragments were gel-purified using the QiaQuik Gel Extraction Kit (Qiagen, Mississauga, ON, Canada) prior to combining them in a three point ligation, thus creating *pDex-KK3RNAi*.

Phenotypic analysis of plant lines

For general growth observations, seeds were imbibed by soaking in water at 4°C in the dark for 48 hours prior to planting on soil. Seeds were sown in Redi-Earth® and plants were maintained at 22°C in a 16:8 hour day: night cycle and monitored for a 50 day period, until seed set. Unless otherwise specified, all plants were treated by germinating seeds in the presence of the appropriate additives (treatment or control). If no differences were detected upon germination, plants were maintained on these plates for further growth observation in the presence of the specific additives. Whenever plants were transferred to additional treatment plates, control plants were simultaneously transferred to control plates to ensure that handling of the plants did

not result in generation of a handling-based artifact. All treatment and control additive preparation protocols are listed in Appendix 2.

Expression profiling of the SALK 051970 T-DNA Insertion Line

Transcript profiles of SALK 051970 line were directly compared with WT in the absence of any external treatment. Plants were cultivated in Redi-earth® (Sun Gro Horticulture, Vancouver, BC, Canada) and cultivated as described. Two biological replicate samples were analyzed, each of which was technically replicated using a dye-swap, and thus a total of four hybridizations were carried out.

RNA extraction for microarray analysis

For each biological replicate, rosette tissue (1 g) was ground under liquid nitrogen and the frozen powder was mixed with 10 mL Trizol reagent (Invitrogen, Burlington, ON, Canada). Cell debris was pelleted by centrifugation at 12 000 rpm (20 minutes; 4°C) and the supernatant was collected. Chloroform (20% v/v) was added to the supernatant, which was quickly vortexed and held at 20°C for 5 minutes, followed by centrifugation at 4 000 rpm for 30 minutes. The aqueous phase was collected and the chloroform extraction was repeated, followed by successive precipitation and resuspension of RNA, first in 0.5 volumes each of isopropanol and 0.8 M sodium citrate, with resuspension in 500 µL RNase-free water, followed by 0.1 volumes 3 M sodium acetate and 2.5 volumes 100% ethanol. The RNA pellet was resuspended in RNase-free water at a concentration of 5 µg RNA/µL following the second precipitation and the RNA quality was assessed using a Bioanalyzer (Agilent Technologies, Mississauga, ON, Canada).

cDNA labeling

Reverse transcription was carried out following a modified RT protocol using SuperScriptII reverse transcriptase (Invitrogen, Burlington, ON, Canada). Each labeling reaction contained 5X First Strand Buffer, 0.5 mM each of dATP, dCTP, and dGTP, 0.05 mM dTTP, 3.75 μM oligo dT anchor primer, 0.01 M DTT, 0.3 μL human spike-RNA, 0.025 nM Cy-dUPT (GE Healthcare, Baie d'Urfe, PQ, Canada), 80 μg total RNA and RNase-free water to a total volume of 37 μL. RNA and oligo d(T) primer (Invitrogen, Burlington, ON, Canada) were denatured at 65°C for five minutes and placed on ice. RNase inhibitor (40 units; Invitrogen, Burlington, ON, Canada) and 400 units SuperScriptII RT (Invitrogen, Burlington, ON, Canada) were added to each reaction. Reverse transcription was carried out at 42°C for two hours and the reaction was stopped by the addition of NaOH (final concentration: 175 mM) and incubation at 65°C for 15 minutes. Labeled cDNA samples were neutralized by the addition of HCl (final concentration 150 mM) and Tris-HCl, pH 7.5 (final concentration: 65 mM). Prior to probe purification, each sample was diluted to a volume of 100 μL. Labeled probe was purified using the QiaQuick PCR purification kit following the manufacturer's protocol (Qiagen, Mississauga, ON, Canada). Paired cDNA samples were pooled, spiked with a Cy5-labeled GFP marker and precipitated overnight at -20°C in 0.1 volumes of sodium acetate and 2.5 volumes 100% ethanol. The precipitated probe was resuspended in 3.5 μL EDTA (10 mM) following a single wash with 70% ethanol.

Hybridization

Labeled probe samples were denatured at 95°C for two minutes, added to 50 μL 48°C hybridization buffer (Ambion #1) and held at 65°C until microarray slides were prepared. Microarray slides (provided by the Treenomix project of Genome British Columbia) were

prepared by incubation in pre-hybridization solution (5X SSC, 0.1% (w/v) SDS, 0.2% (w/v) BSA) while shaking at 48°C for one hour. Slides were then washed twice with distilled water for ten seconds at room temperature, dipped in isopropanol and dried by centrifugation at 2 000 rpm while mounted inside a 50 mL polypropylene tube.

Labeled probes were applied to the microarray slides along the vertical axis of the slide, covered with untreated glass coverslips (Fisher Scientific, Nepean, ON, Canada) and mounted in slide holder cassettes. Hybridization was carried out for 14 hours in a water bath (42°C) while shaking at 40 rpm. Hybridized slides were then washed first in 2X SSC, then in 0.5% (w/v) SDS and twice in 0.5X SSC, 0.5% (w/v) SDS, with each wash carried out at 42°C while shaking at 40 rpm. A final one minute wash in 0.1X SSC wash was carried out at room temperature prior to drying the arrays by centrifugation. The microarrays were then scanned using a Scan Array™ Express model ASCEX00 (Perkin-Elmer, Woodbridge, ON, Canada) scanner, and spot intensities were quantified by ImaGene™ software (BioDiscovery, Marina Del Rey, CA, USA) using 95% laser power and photomultiplier tube set to 50-70%.

Microarray Data Analysis

Raw intensities derived from the ImaGene quantification software were background corrected and normalized using the loess procedure as described by Ehlting et al. (2005). This yielded two $\log_2$-transformed expression ratios comparing the expression of a given gene in the SALK 051970 line to its expression in WT plants.

t-Statistics for each probe and for each replicate ratio were generated using customized scripts for R (The R Development Core Team, www.r-project.org).

Real-time PCR analysis

Three biological replicates each consisting of five 21-day-old plants were cultivated independently as described above, and rosette tissue was harvested and stored at -80°C. Frozen tissue from each replicate was homogenized by vortexing and total RNA was extracted from 100 mg homogenized tissue using the RNeasy Plant Mini Kit (Qiagen, Mississauga, ON, Canada) in combination with the on-column DNase kit (Qiagen, Mississauga, ON, Canada). cDNA was synthesized from 2 μg total RNA using Superscript II (Invitrogen, Burlington, ON, Canada) and the final product was diluted to a total volume of 200 μL. Real-time quantitative PCR was performed using the SYBR Green PCR kit (Qiagen, Mississauga, ON, Canada) and 5 μL cDNA per 20 μL PCR reaction. PCR reactions were carried out in duplicate for each PCR primer pair. Thermo-cycling was carried out using a MJ DNA Engine coupled with a Continuous Fluorescence Detector (MJ Research, Mississauga, ON, Canada) and data were analyzed using Opticon MONITOR Analysis software (MJ Research, Mississauga, ON, Canada). Briefly, average *actin 1* levels for each replicate were calculated based on four amplification reactions. Expression levels for each test gene were obtained from two independent amplification reactions per replicate sample. These values were normalized to the mean *actin 1* (test gene signal / mean *actin 1* signal) expression level and a mean normalized expression level was then calculated for each gene in each replicate. These mean normalized expression levels were then averaged for each of the three biological replicates to generate a mean normalized expression value for each gene. Pair-wise t-tests with a p-value cutoff of 0.05 were used to test for statistically significant differences between the SALK 051970 and WT lines in expression levels for each test gene. All primer sequences used for the quantitative PCR reactions are presented in Appendix 4.

Induction of gene expression using dexamethasone

In order to induce expression of the *MKK3 RNAi* construct in soil-grown transgenic Arabidopsis plants, the plants were sprayed to run-off with a 25 μM dexamethasone solution (25 μM dexamethasone, from a 30 mM stock in 100% ethanol; 0.015% Silwet L-77). Control plants were simultaneously treated with a mock dexamethasone solution (0.83% ethanol; 0.015% Silwet L-77). Seedlings cultivated on ½ MS plates were dexamethasone-treated by immersion of the seedlings in a 25 μM dexamethasone solution (or mock solution as above, for the controls) for a period of 15 minutes, followed by aspiration of the dexamethasone solution. Plates were then sealed with surgical tape (3M) and plants were harvested at the appropriate time point.

RESULTS

PCR analysis of the SALK 051970 T-DNA insertion line

Plants homozygous for the T-DNA insertion in the SALK 051970 line were initially identified by PCR analysis using genomic DNA extracted from mature rosette leaves (Figure 3.4). To verify that the T-DNA insertion in the SALK 051970 line resulted in the elimination of a full-length MKK3 transcript, RT-PCR analysis was carried out using cDNA derived from 21-day old rosette tissue mRNA and the PCR primers MKK3FL F and MKK3-3'UTR-R (Figure 3.4). Samples which did not display a signal following 40 amplification cycles were deemed to be *mkk3*-null mutants (Figure 3.4). To ensure successful cDNA synthesis had occurred for each sample, *actin 1* transcripts were amplified using *actin 1* F and *actin 1* R primers (data not shown). *mkk3* plants were allowed to grow until seed set, following which, seeds were collected and pooled for phenotypic analysis.

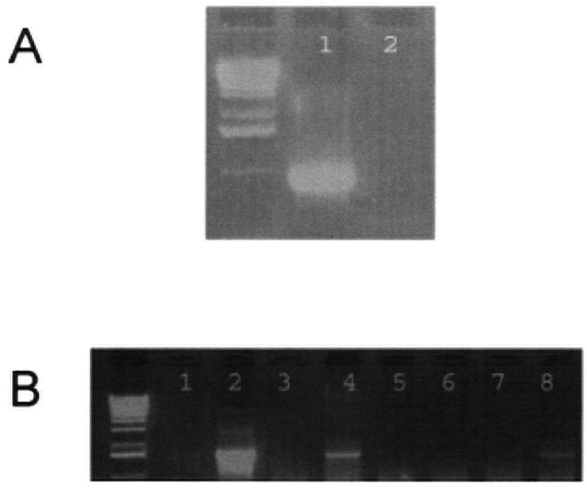

Figure 3.4. PCR characterization of the SALK 051970 T-DNA insertion line. A) Representative PCR analysis using KK3ScreenF and KK3ScreenR primers which flank the T-DNA insertion in the SALK 051970 line and genomic DNA isolated from WT plants (1) and homozygous SALK 051970 plants (2). The absence of a band in lane 2 reflects the inability to successfully amplify across the 5 kb T-DNA insert. B) RT-PCR analysis of cDNA derived from 6 different T4 SALK 051970 plants and MKK3FL-F and MKK3-3'UTR-R primers. This primer set amplifies the complete MKK3 transcript, if present, from the cDNA sample. Lane 1 represents a negative (no template) control. Full length MKK3 amplified from plasmid DNA carrying an MKK3 insertion was used as a positive control (Lane 2). Samples 2 and 6 (lanes 4 and 8) were deemed to be either heterozygous for the T-DNA insertion or WT. Samples 1, 3, 4 and 5, (lanes 3, 5, 6 and 7) were deemed to be homozygous for the T-DNA insertion since a full-length MKK3 transcript was not detected.

Subsequent analyses of transcript levels in transgenic Arabidopsis plants expressing either a full-length *MKK3* or a *MKK3ΔNTF2* variant under the control of the CaMV 35S promoter in the SALK 051970 background revealed that the SALK051970 line continued to show the expression of the N-terminal region of MKK3 (Figure 3.5). This fragment could be detected in all independent SALK 051970 samples tested using the PCR primers (MKK3QRT-F and MKK3QRT-R). Genomic DNA contamination was ruled out since this particular primer set was designed such that the forward primer was situated in exon 2 and the reverse primer was situated in exon 3, targets sites that encompass an 89 base pair intron in the genomic sequence.

Therefore, the possibility remains that the SALK 051970 T-DNA insertion line does not represent a full *mkk3*-null mutant, since it has the potential to produce a C-terminally truncated MKK3 protein.

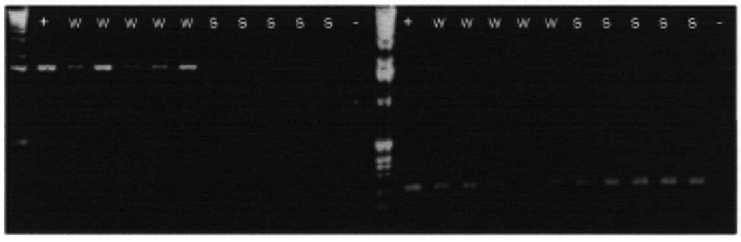

Figure 3.5. RT-PCR analysis of WT and SALK 051970 cDNA. WT (w) and SALK 051970 (s) cDNA samples were analysed using the MKK3FL-F and MKK3-3'UTR-R primer pair which amplifies full-length MKK3 (first half of gel; ~1.6 kb amplicon) and the MKK3QRT-F and MKK3QRT-R pair which amplifies a 225 bp region in the N-terminus of MKK3, upstream of the T-DNA insertion in the SALK 051970 T-DNA insertional mutant line (second half of gel). While a transcript encoding full-length MKK3 is not present in the SALK 051970 samples, a partial transcript extending from the 5' end of the gene to the 5' end of the T-DNA insertion could still be detected, with expression levels visually similar to those observed for full-length MKK3 transcripts in WT samples. Plasmid DNA carrying an MKK3 cDNA insert was used as a positive control (+) while samples with no DNA template added served as negative controls (-).

Phenotypic analysis of the SALK 051970 MKK3 T-DNA insertional mutant

Growth of the SALK 051970 MKK3 T-DNA insertion line was compared with WT plants from germination through to seed set (Table 3.2).

Table 3.2 Comparison of SALK 051970 and WT growth on soil. Seeds were imbibed at 4°C in the dark for 48 hours prior to spreading on Redi-Earth. Over a period of 50 days, the growth characteristics of SALK 051970 and WT plants cultivated at 21°C in a 16:8 hour light: dark lighting regime were recorded.

Age/Trait	3	7	10	14	18	21	24	28	31	35	38	42	45	50
Time to germinate	=													
Time to first leaves		+[1]												
petiole length of largest leaf set at bolting						=								
leaf size		=	=	=	+[2]	=	=	=	=	=	=	=	=	=
leaf shape		=	=	=	=	=	=	=	=	=	=	=	=	=
leaf colour		=	=	=	=	=	=	=	=	=	=	=	=	=
leaf margins								=	=	=	=	=	=	=
stem length						=	=	=	=	=	=	=	=	=
stem colour						=	=	=	=	=	=	=	=	=
overall health in soil	=	=	=	=	=	=	=	=	=	=	=	=	=	=
time to bolting						=	=							
# leaves when bolting						=	=							
bolt length								=	=	=	=	=	=	=
stem length								=	=	=	=	=	=	=
time to flower							=							
# flowers per bolt							=	=	=	=	=	=	=	
flower morphology								=	=	=	=	=	=	
silique length									=	=	=	=	=	=
# of siliques								=	=	=	=	=	=	=
seed set (gross mass per plant)														=
speed of senescence										=	=	=	=	
leaf browning										=	=	=	=	=
stop flowering												=		

Equivalent (=)
+[1] Slight difference but not significant with SALK 051970 plants developing slightly earlier
+[2] Leaves of SALK 051970 plants that developed leaves earlier had slightly larger leaves at this stage

No differences were observed between the SALK 051970 plants and WT, outside of a minor difference in the amount of time between germination and production of the first leaves. In some SALK 051970 plants, the first leaves appeared approximately one day earlier than in WT plants. However, this difference was sporadic and was therefore not examined further.

Similarly, the leaves of some SALK 051970 plants appeared slightly larger than WT in 18-day-old plants, but again this trait appeared in the same plants showing early leaf development and so was not examined in greater detail.

These plant lines were also subjected to a panel of biotic and abiotic treatments to identify phenotypic abnormalities resulting from the T-DNA insertion (Table 3.3).

Table 3.3. Summary of treatment panel to identify phenotypic differences between the SALK 051970 T-DNA insertion line and WT. Seeds were surface-sterilized and plated on ½ MS agar plates containing the described additive. Germination and growth in the presence/absence of treatment was observed and described.

Treatment	Concentration/Description	Result
Jasmonate (JA)	Germination on 1 μM JA	No difference in germination or continued growth
ACC (ethylene)	Germination on 2 μM ACC	No difference in germination or continued growth
ACC (ethylene)	Growth in the dark in presence of 2 μM ACC	No difference in growth
1-naphthylphthalamic acid (NPA)	Germination on 5 μM NPA	No difference in germination or continued growth
IAA (auxin)	Germination on 1 μM IAA	No difference in germination or continued growth
2,4-D (auxin)	Germination on 1 μM 2,4-D	No difference in germination or continued growth
ABA	Germination on 15 and 50 μM ABA	No germination
Kinetin (cytokinin)	Germination on 0.5 μM kinetin	No difference in germination or continued growth
Gibberellin (GA)	Germination on 10 μM GA	No difference in germination or continued growth
Epi-brassinolide (BR)	Germination on 1 μM BR	No difference in germination or continued growth
Salicylic acid (SA)	Germination on 200 μM SA	No difference in germination or continued growth

Treatment	Concentration/Description	Result
SA	Transfer of 10-day old seedlings to 200 μM SA	No difference in growth
Cold	24 hours exposure to 4°C cold treatment	No difference in recovery
Cold	Continued growth at 4°C	No difference in growth
Desiccation	Stop watering bolting plants for a period of 5 days, followed by a return to a normal watering regime	No difference in recovery
NaCl	Germination on 50, 100, 150 and 200 mM NaCl	No difference in germination or continued growth
NaCl	10-day old seedlings transferred to 100, 200 and 300 mM NaCl	No difference
Sucrose	Germination on 2% and 5% sucrose	No difference in germination or continued growth
Ozone	500 ppb for 8 hours followed by a 16 hour recovery period	No difference in ozone sensitivity
Sodium nitroprusside (NO donor; SNP)	Growth following transfer of 10-day old seedlings to ½ MS plates containing 100 μM SNP	No difference in growth
Potassium chloride	Germination on 50 mM KCl	No difference in germination or continued growth
Sorbitol	Germination on 5% sorbitol	No difference in germination or continued growth
LiCl	Germination on 15 mM LiCl	No difference in germination or continued growth
Caffeine	Germination on 2 mM caffeine	No difference in germination or continued growth
Pseudomonas syringae maculicola ES4326 Infection	21-day old rosette leaves were infiltrated with culture at either an OD_{600} of 0.001 (resistance dose) or 0.0001 (susceptibility dose) and plants were monitored for three days	No difference in susceptibility or resistance

Transcriptional profiling of the SALK 051970 MKK3 T-DNA insertion line

Gene expression profiles of both 10- and 21-day old untreated SALK 051970 seedlings were compared with corresponding WT samples using a full transcriptome, 70-mer long oligo microarray. In both experiments, two biological replicates were performed, each containing a dye-swap for technical replication. Unfortunately, for the 10-day old seedling dataset, all hybridizations failed to produce usable signals; the mean percentages of detectable spots three-fold over background were 16.4% for the Cy5 channel and 38.8% for the Cy3 channel. This inefficiency was presumably due to poor hybridization resulting from polysaccharide contamination of the RNA samples (personal communication, Dr. J. Ehlting, IBMP-CNRS, Strasbourg, France).

The hybridizations carried out with cDNA derived from the 21-day old seedlings were more successful, with mean percentages of detectable spots three-fold over background of 65.2% for the Cy5 channel and 55.35% for the Cy3 channel. The 2X2 signal spread plots from these experiments showed that the overall signal spread was uniform, and also suggested that there would not be many significant differences in transcript abundance between the two genotypes (Appendix 3).

Following normalization of the data by loess methods (Materials and Methods), the suggestion that a low number of significant differences in gene expression between the genotypes were substantiated by the overall loess ratio distribution (Appendix 3). This curve appeared to be normally distributed with very few points lying above the normal two-fold expression difference cut-off.

Due to the small number of genes showing >2-fold up-regulation in the SALK 051970 line relative to WT (3 genes), the fold-change cutoff was expanded to include all genes showing at

least a 1.75 fold up-regulation ($\log_2 = 0.7$) but still possessing p-values of 0.05 or smaller. This resulted in a list of 35 up-regulated genes in the SALK line relative to WT (Table 3.4).

Table 3.4. Genes up-regulated in the SALK 051970 MKK3 T-DNA insertion line relative to wild-type. A fold-change cutoff of 1.75 and a p-value cutoff of 0.05 were used to generate this list.

Locus	Annotation	Log_2 (SALK/WT)	p-value
At3g19680	expressed protein	1.4	0.011
At1g14210	ribonuclease	0.9	0.005
At2g45400	dihydroflavonol 4-reductase family	0.8	0.049
At2g21650	myb family transcription factor	0.8	0.002
At1g03820	expressed protein	0.8	0.040
At1g72430	auxin-induced (indole-3-acetic acid induced) protein-related	0.8	0.023
At4g22480	Glycine-rich protein	0.8	0.001
At3g30180	cytochrome p450, putative	0.8	0.017
At4g38840	auxin-induced (indole-3-acetic acid induced) protein, putative	0.8	0.005
At4g04030	hypothetical protein	0.8	0.014
At4g21870	heat shock protein family	0.8	0.004
At1g80710	transducin / WD-40 repeat protein family	0.7	0.041
At1g07610	metallothionein-related protein	0.7	0.003
At1g72470	exocyst subunit EXO70 family	0.7	0.041
At1g02450	expressed protein	0.7	0.002
At5g42040	hypothetical protein	0.7	0.010
At3g44450	expressed protein	0.7	0.021
At1g76870	hypothetical protein	0.7	0.005
At5g08350	expressed protein	0.7	0.002
At3g20020	Protein arginine N-methyltransferase family	0.7	0.022
At2g21220	auxin-induced (indole-3-acetic acid induced) protein, putative	0.7	0.046
At4g24140	hydrolase, alpha/beta fold family	0.7	0.037
At2g43720	expressed protein	0.7	0.003
At5g43640	40S ribosomal protein S15 (RPS15E)	0.7	0.003
At5g54610	Ankyrin-repeat-containing protein-related	0.7	0.036
At4g03210	xyloglucan endotransglycosylase, putative	0.7	0.034
At2g07020	Protein kinase family	0.7	0.001
At5g47170	hypothetical protein	0.7	0.021
At4g38400	expansin protein family (EXPL2)	0.7	0.041
At2g41210	phosphatidylinositol-4-phosphate 5-kinase –related	0.7	0.039
At1g16445	expressed protein	0.7	0.018
At5g67510	60S ribosomal protein L26 (RPL26B)	0.7	0.027
At3g11990	expressed protein	0.7	0.024
At5g39360	expressed protein	0.7	0.010
At3g06770	polygalacturonase, putative	0.7	0.027

Although a larger number of down-regulated genes was detected in the microarray dataset, the fold-change cutoff was again expanded to -1.75 (log_2 = -0.7), for consistency. This yielded a list of 111 genes that were down-regulated in the SALK line relative to WT (Table 3.5).

Table 3.5. Genes down-regulated in the SALK 051970 MKK3 T-DNA insertion line relative to wild-type. A fold-change cutoff of -1.75 and a p-value cutoff of 0.05 were use to generate this list.

Locus	Annotation	Log_2 (SALK/WT)	p-value
At3g48360	expressed protein	-2.6	0.010
At4g27410	no apical meristem (NAM) protein family	-1.8	0.009
At1g19180	expressed protein	-1.6	0.002
At1g80840	WRKY family transcription factor (WRKY40)	-1.5	0.026
At1g40107	putative transposase protein (CACTA-element) transposon	-1.5	0.027
At3g47340	glutamine-dependent asparagine synthetase	-1.5	0.002
At1g69490	no apical meristem (NAM) protein family	-1.4	0.029
At1g11210	expressed protein	-1.4	0.010
At2g26190	expressed protein	-1.4	0.001
At2g38470	WRKY family transcription factor (WRKY33)	-1.3	0.008
At3g07350	expressed protein	-1.3	0.009
At1g21326	hypothetical protein	-1.3	0.003
At4g39060	F-box protein family	-1.3	0.040
At5g03210	expressed protein	-1.2	0.040
At1g27730	salt-tolerance zinc finger protein	-1.2	0.019
At2g46680	homeobox-leucine zipper protein ATHB-7 (HD-ZIP protein ATHB-7)	-1.2	0.008
At2g26190	expressed protein	-1.2	0.028
At3g47340	glutamine-dependent asparagine synthetase	-1.2	0.037
At2g22880	hypothetical protein	-1.2	0.041
At1g53170	ethylene responsive element binding factor 8	-1.2	0.034
At4g23190	serine/threonine kinase - like protein	-1.2	0.011
At5g54490	calcium-binding protein, putative	-1.1	0.006
At5g26340	hexose transporter, putative	-1.1	0.022
At2g36770	glycosyltransferase family	-1.1	0.034
At1g28370	ethylene responsive element binding factor 11, putative (EREBP11)(ERF11)	-1.1	0.014
At4g35770	senescence-associated protein sen1	-1.1	0.008
At3g11410	protein phosphatase 2C (PP2C), putative	-1.0	0.012
At2g23440	expressed protein	-1.0	0.019
At5g22920	PGPD14 protein	-1.0	0.019
At1g35210	expressed protein	-1.0	0.016
At2g22500	mitochondrial carrier protein family	-1.0	0.035
At4g24230	expressed protein	-1.0	0.005
At3g52400	syntaxin of plants SYP122	-1.0	0.006
At1g02660	lipase (class 3) family	-1.0	0.018
At5g04340	C2H2 zinc finger transcription factor –related	-1.0	0.019
At3g52060	expressed protein	-1.0	0.000
At1g21400	branched-chain alpha keto-acid dehydrogenase –related	-1.0	0.004
At3g15210	ethylene responsive element binding factor 4 (ERF4)	-1.0	0.012
At4g31550	WRKY family transcription factor (WRKY11)	-1.0	0.014
At5g42050	expressed protein	-1.0	0.034
At3g28850	expressed protein	-0.9	0.050
At3g48650	expressed protein	-0.9	0.003
At1g61470	hypothetical protein	-0.9	0.035
At4g37710	expressed protein	-0.9	0.002
At5g63790	No apical meristem (NAM) protein family	-0.9	0.000
At2g20670	expressed protein	-0.9	0.012
At3g26220	cytochrome P450 family	-0.9	0.004
At1g77450	No apical meristem (NAM) protein family	-0.9	0.005
At3g10930	expressed protein	-0.9	0.038
At4g11530	serine/threonine kinase-related protein (fragment)	-0.9	0.024
At2g41010	expressed protein	-0.9	0.009
At2g31880	leucine-rich repeat transmembrane protein kinase, putative	-0.9	0.001
At1g21000	expressed protein	-0.9	0.043
At1g80600	acetylornithine aminotransferase, mitochondrial, putative	-0.9	0.014
At1g68670	expressed protein	-0.9	0.021
At5g36925	expressed protein	-0.8	0.010
At1g60190	hypothetical protein	-0.8	0.019
At5g56550	expressed protein	-0.8	0.034

Locus	Annotation	Log_2 (SALK/WT)	p-value
At5g47230	ethylene responsive element binding factor 5 (AtERF5)	-0.8	0.048
At5g56870	glycosyl hydrolase family 35 (beta-galactosidase)	-0.8	0.029
At2g39800	delta 1-pyrroline-5-carboxylate synthetase A (P5CS A) (P5CS1)	-0.8	0.022
At5g46710	expressed protein	-0.8	0.041
At1g20510	4-coumarate:CoA ligase 1 (4-coumaroyl-CoA synthase 1) (4CL1) family	-0.8	0.017
At5g20230	plastocyanin-like domain containing protein	-0.8	0.025
At1g67970	heat shock transcription factor 5 (HSF5)	-0.8	0.005
At2g18200	hypothetical protein	-0.8	0.043
At2g32800	protein kinase family	-0.8	0.006
At1g21670	expressed protein	-0.8	0.005
At5g05860	glucuronosyl transferase-related protein	-0.8	0.021
At1g56600	galactinol synthase, putative	-0.8	0.018
At4g33150	lysine-ketoglutarate reductase/saccharopine	-0.8	0.032
At1g70700	expressed protein	-0.8	0.002
At3g44260	CCR4-associated factor 1-related protein	-0.8	0.008
At1g17990	12-oxophytodienoate reductase, putative	-0.7	0.021
At2g22200	AP2 domain transcription factor	-0.7	0.018
At5g23010	2-isopropylmalate synthase-related; homocitrate synthase-like	-0.7	0.040
At1g20450	dehydrin (ERD10)	-0.7	0.001
At1g45015	expressed protein	-0.7	0.044
At4g37180	cytoskeletal protein –related	-0.7	0.008
At3g15500	no apical meristem (NAM) protein family	-0.7	0.022
At4g23980	auxin response transcription factor (ARF9)	-0.7	0.034
At1g10340	ankyrin repeat protein family	-0.7	0.029
At1g03230	Expressed protein	-0.7	0.022
At2g28200	zinc-finger protein –related	-0.7	0.013
At5g18270	NAM (no apical meristem)-related protein	-0.7	0.016
At1g07430	protein phosphatase 2C (PP2C), putative	-0.7	0.035
At5g19120	conglutin gamma - like protein	-0.7	0.020
At4g11280	1-aminocyclopropane-1-carboxylate synthase 6 (ACC synthase 6) (ACS6)	-0.7	0.002
At3g22720	hypothetical protein	-0.7	0.042
At5g59550	expressed protein	-0.7	0.011
At3g57450	Expressed protein	-0.7	0.002
At2g35900	expressed protein	-0.7	0.004
At1g72940	disease resistance protein (TIR-NBS class), putative	-0.7	0.043
At1g20440	dehydrin (COR47)	-0.7	0.003
At3g26740	light regulated protein –related	-0.7	0.030
At5g06860	polygalacturonase inhibiting protein (PGIP1)	-0.7	0.008
At1g68520	CONSTANS B-box zinc finger family protein	-0.7	0.016
At1g08890	sugar transporter family	-0.7	0.026
At4g23180	serine/threonine kinase -related protein	-0.7	0.015
At2g45940	hypothetical protein	-0.7	0.004
At5g52110	expressed protein	-0.7	0.011
At5g15100	auxin efflux carrier protein family	-0.7	0.033
At3g02040	expressed protein	-0.7	0.025
At3g60170	hypothetical protein	-0.7	0.033
At2g30250	WRKY family transcription factor (WRKY25)	-0.7	0.013
At3g10020	expressed protein	-0.7	0.012
At2g12920	reverse transcriptase-related	-0.7	0.038
At1g58270	expressed protein	-0.7	0.034
At3g15630	Expressed protein	-0.7	0.002
At4g27260	GH3 like protein	-0.7	0.026
At5g46540	multidrug resistance protein, putative	-0.7	0.012

Real-time PCR validation of SALK 051970/WT microarray results

To verify that the genes identified by the microarray analysis were true transcriptional differences between the SALK 051970 T-DNA insertion line and WT, nine genes were selected

for analysis by real-time quantitative PCR using cDNA derived from three independent biological replicates of 21-day old, untreated rosette tissue. These genes were selected to include genes showing a range of expression differentials and p-values. Of the nine genes analysed by this method, eight showed similar expression patterns to the microarray analysis (Figure 3.6). One gene, At4g04030, yielded an inconsistent result. However, this gene is apparently expressed at very low levels, and produced microarray signals only marginally above the background cutoff used in the array data analysis. Such weak signals tend to produce unreliable results, which may explain my inability to replicate the microarray pattern for this gene. Given the high concordance between the microarray results and the independent quantitative RT-PCR verification, I felt that further *in silico* analyses of the differentially regulated gene sets were justified.

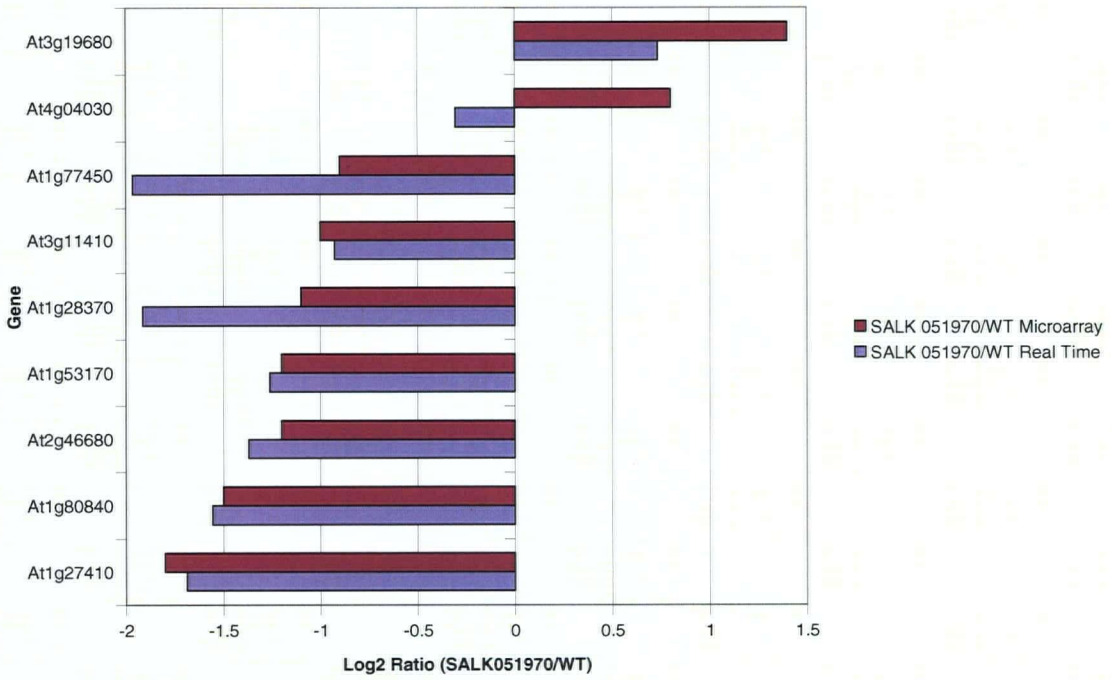

Figure 3.6. Real-time PCR validation of SALK 051970/WT microarray data. Expression profiles for each gene were determined using quantitative real-time PCR and cDNA from three independent biological replicates of 21-day-old rosette tissue of both WT and SALK 051970 plants. Data are reported as Log_2 ratios SALK 051970/WT (mean signal strength normalized against *actin 1* content).

Identification of over-represented CAREs in the promoter regions of differentially expressed genes

Genes whose expression can be influenced by the absence of full-length MKK3 would be expected to be regulated by transcription factors whose activity and/or abundance in the cell is being controlled, directly or indirectly, by MKK3. The ability of such trans-acting factors to affect the MKK3 promoter typically relies upon recognition of cis-elements in the promoter, and the prediction is that groups of co-regulated genes will display some degree of commonality in the pattern of cis-elements (CAREs) located in their promoter regions. I therefore analyzed the

promoters of all the genes that displayed differential expression patterns in the MKK3 T-DNA line, relative to WT, to establish whether any known CAREs were over-represented within this set of promoters (Athena; http://www.bioinformatics2.wsu.edu/cgi-bin/Athena/cgi/home.pl). This analysis identified three CAREs that appeared to be over-represented in the list of up-regulated genes (Table 3.6).

Table 3.6. Athena output from the up-regulated gene list generated from the SALK 051970/WT microarray experiment. 1500 base pairs upstream of the translation initiation codon of each gene were examined using Athena to detect over-representation of known CAREs.

Motif Name	Frequency of occurrence in gene list	Frequency of occurrence in genome	p-value
CARGCW8GAT	82%	61%	0.004
Ibox promoter motif	57%	39%	0.027
TATA-box Motif	91%	79%	0.048

A similar analysis of the down-regulated genes in the SALK 051970/WT microarray experiment found that 25 CAREs were over-represented in this dataset. Interestingly, hormone- and stress-associated CAREs were included in this list, with the ABRE (ABA), ARF (auxin), AtHB6 (general hormone), DREB (salt and drought), Myb4 (environmental stress), and W-box (WRKY/stress) all being over-represented (Table 3.7).

Table 3.7. Athena output from the down-regulated gene list generated from the SALK 051970/WT microarray experiment. The 1500 bp sequence upstream of the translation initiation codon of each gene was examined using Athena for the over-representation of known CAREs.

Motif Name	Frequency of occurrence in gene list	Frequency of occurrence in genome	p-value
ABRE-like binding site motif	47%	22%	1.000E-07
CACGTGMOTIF	37%	16%	1.000E-06
GADOWNAT	28%	9%	1.000E-06
CARGCW8GAT	84%	61%	0.00001
ACGTABREMOTIFA2OSEM	35%	15%	0.00001
T-box promoter motif	68%	53%	0.00100
Ibox promoter motif	60%	39%	0.00100
DREB1A/CBF3	16%	7%	0.00100
Z-box promoter motif	11%	2%	0.00100
MYB4 binding site motif	82%	70%	0.00300
GAREAT	68%	55%	0.00300
DRE core motif	34%	22%	0.00400
UPRMOTIFIAT	8%	3%	0.00900
TGA1 binding site motif	8%	3%	0.00900
TATA-box Motif	92%	79%	0.01000
W-box promoter motif	75%	65%	0.01000
SV40 core promoter motif	33%	19%	0.01000
ARF binding site motif	48%	37%	0.01200
MYB1AT	87%	78%	0.01600
ATHB2 binding site motif	20%	13%	0.02000
CCA1 binding site motif	37%	28%	0.02600
ATHB6 binding site motif	9%	4%	0.02600
UPRE2AT	2%	0%	0.03400
MYB1LEPR	25%	18%	0.03600
UPRMOTIFIIAT	7%	3%	0.04400

Gene ontology of deferentially expressed genes

To gain further insight into the possible biological role(s) of MKK3, gene ontology (GO) reports were generated from the lists of genes that were differentially expressed in the SALK 051970 line relative to WT (TAIR; www.arabidopsis.org; (Gene Ontology Consortium, 2004); Figure 3.7 and 3.8).

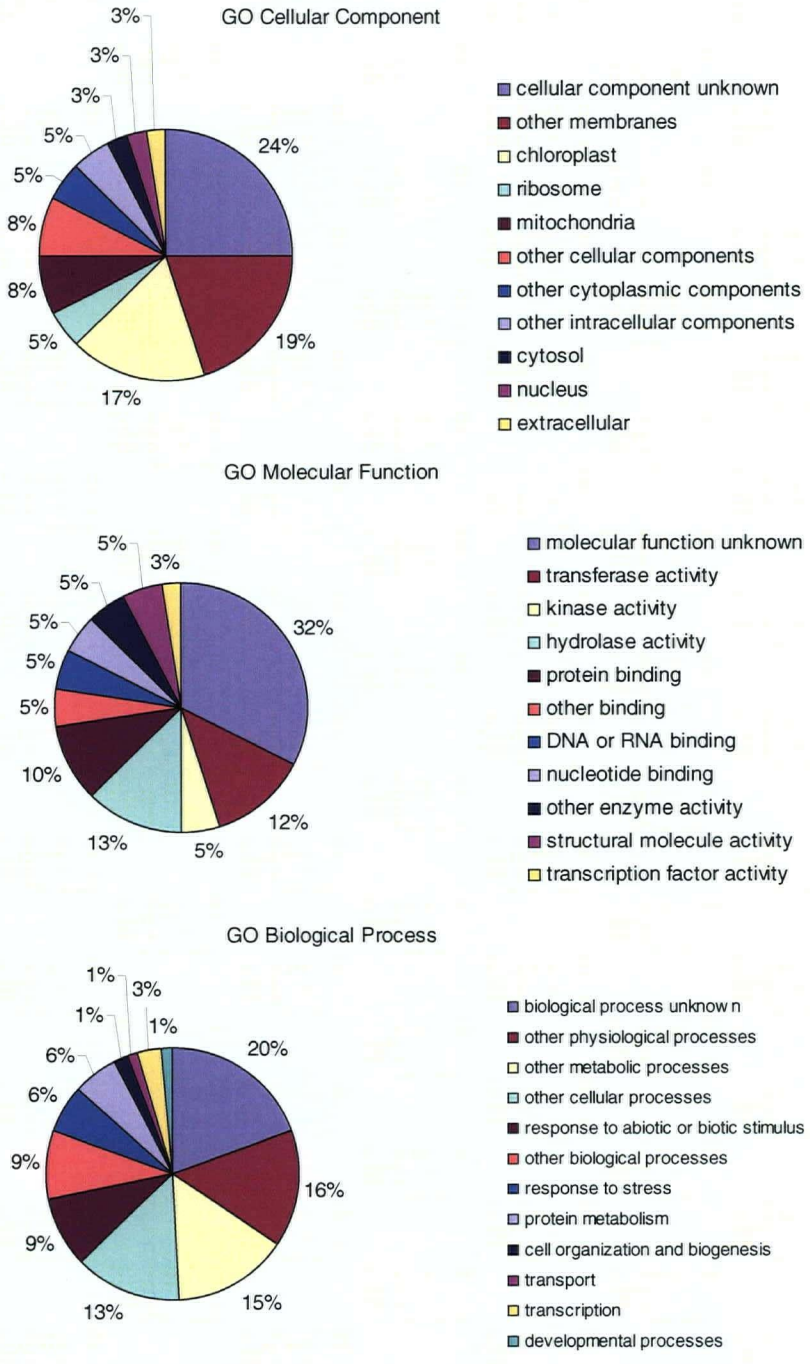

Figure 3.7. GO analysis of genes up-regulated genes in the SALK 051970 line relative to WT. Gene ID's of up-regulated regulated genes were analysed using Gene Ontology by TAIR (www.arabidopsis.org). Pie charts represent the total gene counts for each GO term.

In each category (cellular component, biological process and molecular function) the majority of genes were placed into "unknown" or "other" categories. For the up-regulated genes, a large portion of the encoded proteins contain putative chloroplast and/or mitochondrial target peptides, indicating that these gene products are likely to be directed to organelles that are known to have roles in controlling the status of the cell during stress responses (Figure 3.7). These data are in agreement with the biological process GO analysis, where 9% of genes were annotated as having a role in the response to environmental stress (Figure 3.7). This group of genes comprised the largest category of genes for which a biological process apart from an "other" classification could be made.

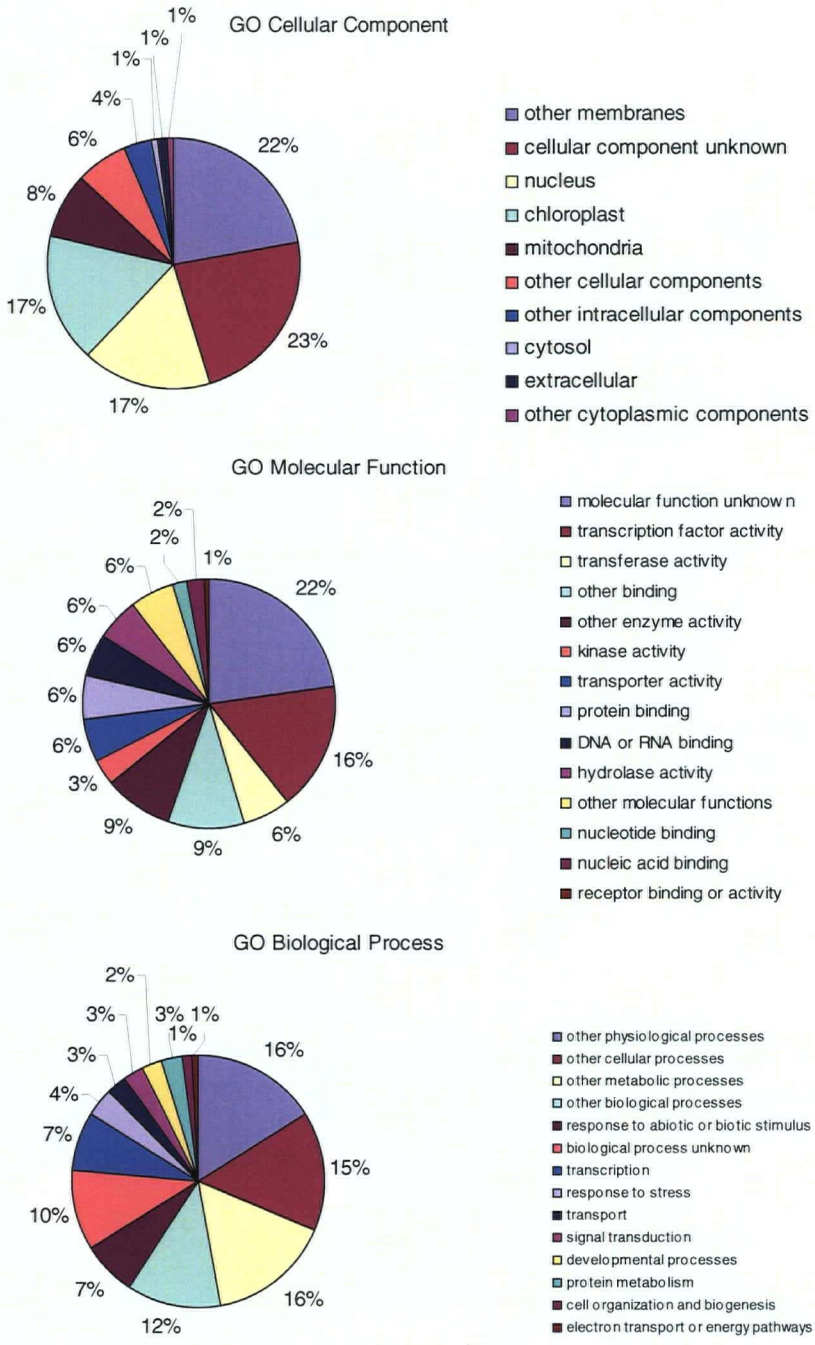

Figure 3.8. GO analysis of genes down-regulated genes in the SALK 051970 line relative to WT. Gene ID's of down-regulated regulated genes were analysed using Gene Ontology by TAIR (www.arabidopsis.org). Pie charts represent the total gene counts for each GO term.

As with the up-regulated genes, the major classifications in all of the GO analyses of down-regulated genes were "unknown" or "other." With respect to protein localization, the largest

class of down-regulated gene products were reported to be targeted to the nucleus (17%) and the chloroplast (17%; Figure 3.8). Mitochondrial proteins accounted for 8% of the gene products. The increase in abundance of nuclear targeted proteins in the list of down-regulated genes relative to that of up-regulated genes is due to the relative abundance (16%) of transcription factors in this group. Not only is this a further indication that MKK3 activity plays a role in controlling transcriptional programming in plants, but it is also of interest because transcription factors are known substrates for MAPKs (Figure 3.8). A link to stress signaling was also evident, since 15% of the genes were annotated as being involved in the response to abiotic or biotic stimuli (Figure 3.8).

To add more significance to the GO analysis, Athena was used to analyze the GO terms assigned to each differentially expressed gene and identify GO terms that were over-represented relative to their occurrence in the genome. In the up-regulated gene list, three genes were identified as being auxin-responsive (At1g72430, At2g21220 and At4g38840; Table 3.8), a frequency that was significantly higher than the occurrence of genes annotated as auxin-responsive within the full genome. Although some other GO terms were also found to be over-represented, many of these were only associated with a single gene, which makes it difficult to assess the significance of the association. Six of the down-regulated genes have been annotated as being involved in plant development (Table 3.8).

Table 3.8. Athena output for genes up-regulated in the SALK 051970 line relative to WT. Gene ID's of genes up-regulated in the SALK 051970 line relative to WT were examined by Athena to identify GO terms that were over-represented in the gene list relative to their frequency in the genome.

GO Term	Number of genes in subset	p-value	GO ID
response to auxin stimulus	3	1.00E-03	9733
methyltransferase activity	2	1.00E-03	8168
endoribonuclease activity	1	1.00E-02	4521
proteasome regulatory particle (sensu eukarya)	1	1.00E-02	5838
protein amino acid methylation	1	1.00E-02	6479
peptidase activity	2	1.00E-02	8233
protein methyltransferase activity	1	1.00E-02	8276
cell wall organization and biogenesis (sensu magnoliophyta)	1	1.00E-02	9664
oxidoreductase activity, acting on CH-OH group of donors	1	1.00E-02	16614
xyloglucan:xyloglucosyl transferase activity	1	1.00E-02	16762
response to copper ion	1	1.00E-02	46688

Down-regulated genes were also found to have over-represented GO terms that correspond to treatments to which the MKK3 promoter was earlier found to respond (Chapter 2), with the four ABA-responsive genes being of particular interest in this regard (Table 3.9). These analyses indicated that 24 genes encoded transcription factors. A link between the down-regulated genes and environmental stresses was illustrated by both methods of GO analysis, with the Athena analysis indicating an over-representation of gene products associated with responses to water deprivation (Table 3.9).

Table 3.9. Athena output for genes down-regulated in the SALK 051970 line relative to WT. Gene ID's of genes down-regulated in the SALK 051970 line relative to WT were examined by Athena to identify GO terms that were over-represented in the gene list relative to their frequency in the genome.

GO Term	Number of genes in subset	p-value	GO ID
transcription factor activity	24	1.00E-07	3700
development	6	1.00E-04	7275
response to water deprivation	4	1.00E-04	9414
response to abscisic acid stimulus	4	1.00E-04	9737
transcriptional repressor activity	3	1.00E-04	16564
response to water	2	1.00E-03	9415
acetylornithine transaminase activity	1	1.00E-02	3992
ribonuclease activity	2	1.00E-02	4540
saccharopine dehydrogenase activity	1	1.00E-02	4753
sugar porter activity	3	1.00E-02	5351
binding	4	1.00E-02	5488
regulation of transcription, DNA-dependent	13	1.00E-02	6355
protein serine/threonine phosphatase complex	2	1.00E-02	8287
RNA modification	2	1.00E-02	9451
response to external stimulus	1	1.00E-02	9605
response to absence of light	1	1.00E-02	9646
response to sucrose stimulus	2	1.00E-02	9744
lysine-ketoglutarate reductase activity	1	1.00E-02	10010
aluminum ion transport	1	1.00E-02	15690
delta1-pyrroline-5-carboxylate synthetase activity	1	1.00E-02	17084
lysine catabolism	1	1.00E-02	19477
glutamate catabolism to ornithine	1	1.00E-02	19555
transferase activity, transferring acyl groups, acyl groups converted into alkyl on transfer	1	1.00E-02	46912

Auxin-responsive genes differentially expressed in the SALK 051970 line relative to WT

Because the MKK3 promoter is known to be activated by auxin exposure (Figure 2.12), the SALK 051970/WT microarray gene lists were queried for the presence of auxin-responsive genes. This analysis yielded eight genes that were either directly annotated as auxin-responsive, or possessed an auxin-responsive GO annotation. Three of these loci encode small auxin up-response proteins (SAURs; At1g72430, At4g38840, At2g21220), one encodes a PINOID

binding protein (PBP; At5g54490), and one encodes a putative auxin efflux carrier protein (At5g15100). ACS6 (At4g11280), an auxin biosynthetic enzyme (At4g27260) and an auxin-response transcription factor (ARF9; At4g23980) were also found in the list. The promoter regions of these genes, together with the MKK3 promoter (At5g40440), were compared to identify overlapping CAREs. All nine genes contained MYB1AT and MYB4 binding sites, eight of the nine contained GAREAT elements, seven contained ARF and T-box elements, and six contained W-Boxes (Table 3.10). This strong pattern of common CAREs suggests that these genes are co-regulated by auxin through multiple trans-acting factors.

Table 3.10. CAREs present in auxin responsive genes found to be differentially regulated in the SALK 051970 MKK3 T-DNA insertion line.

	ARF	MYB1AT	MYB4	W-Box	T-Box	GAREAT
At1g72430	/	X	X	/	/	X
At4g38840	X	X	X	X	X	X
At2g21220	X	X	X	X	X	X
At5g54490	/	X	X	/	X	X
At4g11280	X	X	X	X	/	X
At5g15100	X	X	X	/	X	/
At4g27260	X	X	X	X	X	X
At4g23980	X	X	X	X	X	X
At5g40440/ *MKK3*	X	X	X	X	X	X

Abscisic acid-responsive genes differentially expressed in the SALK 051970 line relative to WT

Because the *MKK3* promoter is also responsive to ABA (Figure 2.10), the microarray dataset was queried for ABA-responsive genes. Six genes were found to be associated with ABA signaling (At2g46680, At3g11410, At3g15210, At2g39800, At1g20450, At1g20440) and these encode a leucine zipper transcription factor (At2g46680), a protein phosphatase 2C (PP2C; At3g11410), an ERF transcription factor known to inhibit the JA-mediated response (AtERF-4;

At3g15210), *delta*-1-pyrroline-5-carboxylate synthetase (P5CS1; At2g39800), and two dehydrins (ERD10; At1g20450 and COR47; At1g20440) both of which can be induced by both cold and ABA stress. Promoter regions of these genes and *MKK3* (At5g40440) were examined using Athena and seven common CAREs were identified (Table 3.11). All promoters contained MYB1AT binding sites, six of seven contained CARGCW8GAT and GAREAT motifs, five encoded AtMYC2 and T-box elements and four contained W-boxes and ABRE motifs.

Table 3.11. CAREs present in ABA responsive genes found to be differentially regulated in the SALK 051970 MKK3 T-DNA insertion line.

	AtMYC2	CARGCW8GAT	MYB1AT	GAREAT	T-Box	W-Box	ABRE
At2g46680	X	X	X	X	X	X	/
At3g11410	X	X	X	X	/	X	X
At3g15210	X	X	X	X	X	X	X
At2g39800	/	X	X	X	X	/	/
At1g20450	X	X	X	/	X	/	X
At1g20440	X	X	X	X	/	/	X
At5g40440/ *MKK3*	/	/	X	X	X	X	/
At1g27730	/	/	X	/	X	X	/

Salt- or drought-induced genes differentially expressed in the SALK 051970 line relative to WT

The distinction between salt-and drought-induced genes is often not clear. Many genes whose expression is influenced by one treatment will be induced by the other. Because of this, I chose to examine all salt- and drought-induced genes that were identified by the microarray study as a group. This list comprised five genes, and with the exception of At1g27730, all were also contained within the ABA-responsive gene list. At1g27730, encodes the salt-tolerance, zinc-finger transcription factor, ZAT10. In view of the strong biological relationships between ABA and drought, ZAT10 was included in the ABA-responsive gene analysis (Table 3.12).

Heat-induced genes differentially expressed in the SALK 051970 line relative to WT

Although the *MKK3* promoter is induced by heat shock (Figure 2.11), only two heat-associated genes were differentially expressed in the SALK 051970 line relative to WT. Both of these genes encoded heat-shock proteins, for which there are no additional annotations. Given the lack of experimental data surrounding these genes, further *in silico* analyses were not performed.

Development-associated genes differentially expressed in the SALK 051970 line relative to WT

Among the genes transcriptionally affected in the T-DNA insertion line, seven have been assigned GO annotations associated with plant development. Five of these genes (At4g27410 (RD26), At1g69490 (NAP), At1g77450, At3g15500 (NAC3) and At5g18270) encode NAM family transcription factors, a gene family that is most notably involved in various aspects of floral development. The remaining two genes encode proteins involved in cell wall remodeling (At4g38400/AtEXLA2) and senescence (At4g35770/SEN1).

The promoter regions of these development-associated genes and *MKK3* (At5g40440) were studied using Athena to identify CAREs common to the majority of genes (Table 3.12).

Table 3.12. Differentially regulated developmentally associated genes in the SALK 051970 line relative to WT.

	ABRE-like	CARGCW8GAT	MYB4	Myb1AT	T-box	W-box	BoxII
At4g27410	X	X	X	X	X	/	X
At1g69640	X	X	X	X	/	X	X
At1g77450	X	X	X	X	X	X	X
At3g15500	X	/	X	X	X	/	X
At5g18270	X	X	X	X	X	X	X
At4g38400	X	X	X	X	X	X	X
At4g35770	/	/	/	/	X	/	/
At5g40440/ *MKK3*	/	/	X	X	X	X	X

The Myb1AT, MYB4 and T-Box, and BoxII elements were contained within the promoters of seven genes, with the ABRE-like motif lying in six out of eight promoters and CARGCW8GAT and W-box motifs were situated in five promoters (Table 3.12). The promoter of the SEN1 gene (At4g35770) contained only one common promoter element, which was the pathogen- and ABA-associated T-box element found in most genes identified by this study as being associated with MKK3 signaling.

Genes encoding transcription factors differentially expressed in the SALK 051970 line relative to WT

Of the known targets of eukaryotic MAPK signaling modules, several are transcription factors. Interestingly, in my microarray analysis 29 out of 148 (20%) of the differentially regulated genes were annotated either directly as a transcription factor or as being involved in transcription (Table 3.13).

Table 3.13. Differentially regulated genes encoding transcription-related proteins in the SALK 051970 line relative to WT.

Locus	Annotation	Log_2 (SALK/WT)	p-value
At2g21650	Myb family transcription factor	0.8	0.002
At1g02450	expressed protein	0.7	0.002
At2g30250	WRKY family transcription factor (WRKY25)	-0.7	0.013
At1g68520	CONSTANS B-box zinc finger family protein	-0.7	0.016
At5g18270	NAM (no apical meristem)-related protein	-0.7	0.016
At2g28200	zinc-finger protein–related	-0.7	0.013
At4g23980	auxin response transcription factor (ARF9)	-0.7	0.034
At3g15500	no apical meristem (NAM) protein family	-0.7	0.022
At4g37180	cytoskeletal protein–related	-0.7	0.008
At2g22200	AP2 domain transcription factor	-0.7	0.018
At3g44260	CCR4-associated factor 1-related protein	-0.8	0.008
At1g67970	heat shock transcription factor 5 (HSF5)	-0.8	0.005
At5g47230	AtERF5	-0.8	0.048
At1g68670	expressed protein	-0.9	0.021
At1g77450	no apical meristem (NAM) protein family	-0.9	0.005
At5g63790	no apical meristem (NAM) protein family	-0.9	0.000
At1g61470	hypothetical protein	-0.9	0.035
At4g31550	WRKY family transcription factor (WRKY11)	-1.0	0.014
At3g15210	AtERF4	-1.0	0.012
At5g04340	C2H2 zinc finger transcription factor-related	-1.0	0.019
At1g28370	AtERF11	-1.1	0.014
At1g53170	AtERF8	-1.2	0.034
At2g46680	homeobox-leucine zipper protein ATHB-7	-1.2	0.008
At1g27730	salt-tolerance zinc finger protein	-1.2	0.019
At2g38470	WRKY family transcription factor (WRKY33)	-1.3	0.008
At1g69490	no apical meristem (NAM) protein family	-1.4	0.029
At1g80840	WRKY family transcription factor (WRKY40)	-1.5	0.026
At4g27410	no apical meristem (NAM) protein family	-1.8	0.009
At3g48360	expressed protein	-2.6	0.010

Of the 29 transcription factors, several belonged to larger gene families, including six NAM family members, four ERFs and four WRKY family members. All of these were down-regulated in the T-DNA insertion line. Included in the list of over-represented CAREs were the same elements found in the promoters of the hormone-related genes that were differentially regulated in the MKK3 T-DNA insertion line (Table 3.14). Specifically, the W- and T-box elements, in addition to the GAREAT, MYB4, CARGCW8GAT and ABRE motifs were over-

represented in the promoters of differentially regulated transcription factors (Table 3.14). Also included in this list were the CACGTG motif and the DRE binding sites. The CACGTG motif, which is also known as the G-box, is known to be associated with a variety of plant gene expression conditions, including development, ABA, and pathogen responses (Menkens et al., 1995). The DRE core motif has been associated with dehydration and other abiotic stress responses (Narusaka et al., 2003).

Table 3.14. Athena output for differentially regulated transcription factors.

Motif Name	Frequency of occurrence in gene list	Frequency of occurrence in genome	p-value
CACGTGMOTIF	50%	16%	0.00100
DRE core motif	50%	22%	0.00100
DREB1A/CBF3	26%	7%	0.00100
GCC-box promoter motif	23%	6%	0.00200
ACGTABREMOTIFA2OSEM	36%	15%	0.00300
ATHB2 binding site motif	33%	13%	0.00300
ABRE binding site motif	20%	5%	0.00300
GADOWNAT	26%	9%	0.00500
SV40 core promoter motif	40%	19%	0.00700
TATA-box Motif	96%	79%	0.00800
GBOXLERBCS	13%	2%	0.00900
ABRE-like binding site motif	50%	22%	0.01000
MYB4 binding site motif	90%	70%	0.01100
ABFs binding site motif	13%	3%	0.01900
CARGCW8GAT	80%	61%	0.02200
UPRMOTIFIIAT	13%	3%	0.02200
GAREAT	73%	55%	0.03300
MYB1LEPR	33%	18%	0.03800
Gap-box Motif	23%	11%	0.04500
ABREATRD22	10%	2%	0.04500
T-box promoter motif	70%	53%	0.05000
W-box promoter motif	80%	65%	0.05000
Z-box promoter motif	10%	2%	0.05000
ARF binding site motif	53%	37%	0.05000

Characterization of transgenic Arabidopsis plants expressing *MKK3* variants

Transgenic Arabidopsis plants expressing either a full-length *MKK3* variant containing a C-terminal triple hemagglutinin (HA) tag and a poly-histidine (6XHis) tag, or a variant of *MKK3* from which the *NTF2* coding sequences had been removed, but also containing a C-terminal triple hemagglutinin (HA) tag and a poly histidine (6XHis) tag, were created in the SALK 051970 background via the floral dip method (Materials and Methods).

The *MKK3-3HA* and *MKK3ΔNTF2-3HA* transgenes were placed under the control of the CaMV 35S promoter. The total level of *MKK3* expression in each transgenic line was determined using quantitative real-time PCR. The highest degree of over-expression of each variant was 1.8 fold for the *MKK3ΔNTF2-3HA* construct and 2.2 fold for the *MKK3-3HA* construct. This inability to more strongly over-express *MKK3* was unexpected, because genes whose expression is driven by the CaMV35S promoter in Arabidopsis are typically highly expressed. Nevertheless, since there was some degree of over-expression of *MKK3* sequences, the phenotypic consequences were examined in some detail.

It had been anticipated that no MKK3 signal would be detected in the SALK 051970 T-DNA insertion line. However, the results from this RT-PCR analysis using internal primers MKK3QRT-F and MKK3QRT-R, which target the region upstream (5') of the T-DNA insertion, revealed that a truncated *MKK3* transcript could still be detected, even though the full-length *MKK3* transcript has been eliminated by the SALK 051970 T-DNA insertion.

The T-DNA insertion in the SALK 051970 line lies within exon 7 of the sequences encoding MKK3, at a point immediately between the kinase and NTF2 domains (Figure 2.2). Although unlikely, the possibility exists that a truncated protein could be synthesized from this aberrant

transcript. If this were indeed the case, the SALK 051970 line would essentially represent a plant which has diminished ability to signal through MKK3 in an NTF2 domain-dependent fashion.

To quantify this phenomenon, total *MKK3* expression levels in the SALK 051970 insertion and WT lines were examined by real-time PCR using the MKK3QRT-F and MKK3QRT-R primer set (Figure 3.9). These analyses revealed that there was no statistically significant difference between the expression levels of this portion of the *MKK3* transcript in either genotype ($\alpha<0.05$).

To establish whether a truncated MKK3 protein was, in fact, being produced in the T-DNA insertion line, antibodies were raised in rabbits against synthetic N-terminal MKK3 peptides. While the resulting antisera were capable of detecting relatively high levels (>200 ng) of recombinant MKK3 protein on western blots, they were not able to detect endogenous MKK3 in protein extracts from either WT or SALK 051970 plants, even with maximum loading of the PAGE gels.

Since the SALK 051970 T-DNA insertion line is the only publicly available line carrying an insertion in the sequences encoding MKK3, I next generated transgenic plants expressing a dexamethasone-inducible RNAi construct, in an attempt to examine the effect of eliminating MKK3 signaling via this gene silencing mechanism.

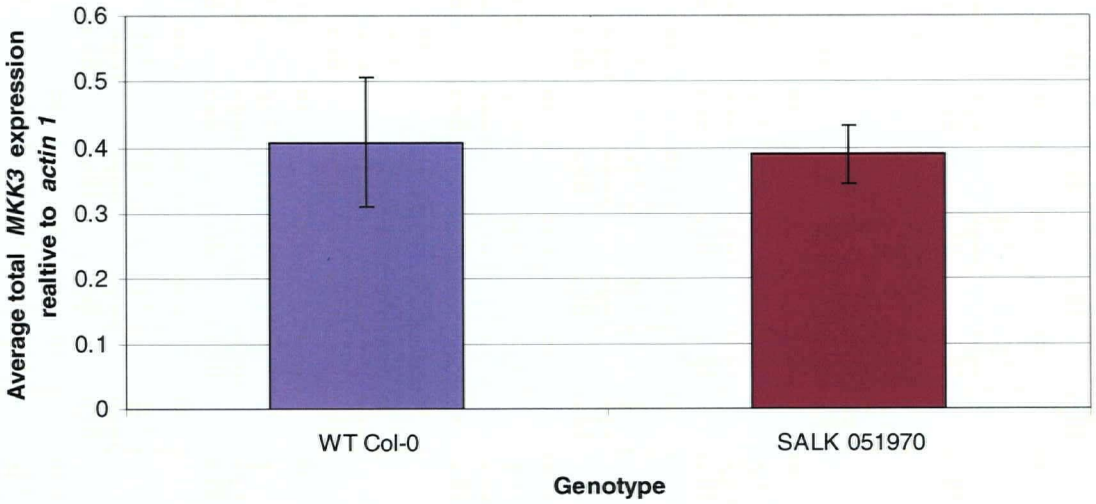

Figure 3.9. Real-time PCR analysis of expression of the 5' region of *MKK3* in the SALK 051970 T-DNA insertional mutant line. Real-time PCR was carried out using MKK3QRT-F and MKK3QRT-R primers and cDNA derived from two biological replicates each consisting of RNA extracted from a pool of five plants for each genotype.

Complementation of the SALK 051970 MKK3 T-DNA insertion

Since specific genes could be shown to have altered expression in the SALK 051970 T-DNA insertion line, it was clear that the T-DNA insertion event had affected certain transcription-associated processes. However, given the uncertainty surrounding the presence, level or functionality of a truncated MKK3 protein in these plants, it remained unclear whether these changes were a consequence of expression of such a truncated MKK3, or a consequence of a complete loss of MKK3. A classic genetic method of addressing this question is to attempt to complement the new phenotype (i.e. in this case, the altered transcript profile) by ectopically expressing a fully functional version of the affected gene. Since even expression of a truncated MKK3 protein would represent a genetic lesion, insofar as the putatively expressed MKK3 would clearly lack its usual NTF2 domain, I extended this complementation exercise to include over-expression of either a full-length version of *MKK3*, or of an NTF2-deleted (*MKK3ΔNTF2*) variant. Three independent replicates each comprising cDNA derived from five plants, from each

of two independent transgenic lines expressing either the *MKK3-3HA* or the *MKK3ΔNTF2-3HA* transgene were cultivated on soil, together with WT plants, for a period of 21-days, at which time all the rosette leaves were harvested. RNA was extracted from each replicate (i.e. six RNA samples for each construct were examined) and duplicate quantitative RT-PCR reactions were carried out using gene-specific primers for each of 22 genes selected from the microarray up- and down-regulated gene lists. Datasets for both biological replicates agreed closely and the results for the highest over-expression line for each MKK3 variant are summarized in Table 3.15.

Seven categories of gene responses were identified from these analyses:

1. Genes down-regulated in the SALK 051970 T-DNA insertion line that were restored to WT levels in both over-expression lines.
2. Genes down-regulated in the SALK 051970 T-DNA line that were up-regulated relative to WT in both over-expression lines.
3. Genes down-regulated in the SALK 051970 T-DNA line that were complemented differentially in the two over-expression lines.
4. Genes down-regulated in the SALK 051970 T-DNA line that were not complemented by either over-expression construct.
5. Genes up-regulated in the SALK 051970 T-DNA line that were not complemented by either over-expression construct.
6. Genes up-regulated in the T-DNA line that were complemented differentially in the two over-expression lines.
7. Genes up-regulated in the SALK 051970 T-DNA insertion line that were restored to WT levels in both over-expression lines.

Table 3.15. Expression profiling of MKK3-related genes in full-length *MKK3* and *MKK3ΔNTF2* over-expression lines in the SALK 051970 background. Expression profiles of genes in the over-expression lines were generated using real-time PCR using cDNA derived from three biological replicates for each line. Expression profiles are reported as $\log_2$ ratios of the expression level of a given gene in a given over-expression line relative to the expression level in WT. **dNTF2** = *MKK3ΔNTF2* over-expression lines; **3HA** = full-length MKK3 over-expression lines. The expression differential for each gene in the original SALK 051970 line (**KK3 T-DNA**) is also shown ($\log_2$ ratios); these values were derived from the microarray comparison of transcripts in the SALK 051970 line relative to WT. p-values <0.05 indicate that the average mean expression levels were statistically different between plant lines.

Genes that were down-regulated in the T-DNA line that showed WT expression levels in both over-expression lines

Gene	Description	dNTF2	p-value	3HA	p-value	KK3 T-DNA
At3g48360	Speckle-type POZ protein - Regulation of transcription	0.60	0.833	0.16	0.719	-2.60
At4g27410	No apical meristem (NAM) protein family (RD26)	-0.09	0.294	-0.50	0.103	-1.80
At1g80840	WRKY family transcription factor (AtWRKY40)	1.75	0.073	0.90	0.246	-1.50
At4g39060	F-box protein family	0.08	0.101	0.57	0.457	-1.30
At2g46680	homeobox-leucine zipper protein ATHB-7	-0.53	0.871	-0.50	0.439	-1.20
At1g53170	ethylene responsive element binding factor 8	0.15	0.092	0.97	0.778	-1.20

Genes that were down-regulated in the T-DNA line that are now up-regulated relative to WT in both over-expression lines

Gene	Description	dNTF2	p-value	3HA	p-value	KK3 T-DNA
At3g52400	syntaxin of plants SYP122	**2.13**	0.010	**1.66**	0.008	-1.00
At3g15210	ethylene responsive element binding factor 4 (ERF4)	**0.69**	0.003	**0.48**	0.043	-1.00
At4g31550	WRKY family transcription factor (AtWRKY11)	**1.45**	0.002	**1.22**	2.6E-04	-1.00
At2g38470	WRKY family transcription factor (AtWRKY33)	**3.56**	2.6E-04	**2.93**	0.009	-1.30
At1g27730	salt-tolerance zinc finger protein (ZAT10/STZ)	**2.57**	8.1E-05	**1.95**	2.6E-05	-1.20
At1g28370	ethylene responsive element binding factor 11 (ERF11)	**2.48**	0.001	**1.73**	0.001	-1.10
At3g11410	protein phosphatase 2C (PP2C), putative	**0.64**	0.020	**0.82**	0.038	-1.00

Genes that were down-regulated in the T-DNA line that were complemented differentially in the over-expression lines

Gene	Description	dNTF2	p-value	3HA	p-value	KK3 T-DNA
At1g69490	No apical meristem (NAM) protein family (NAP)	0.45	0.074	**1.81**	1.9E-04	-1.40
At5g04340	c2h2 zinc finger transcription factor –related	**1.02**	0.023	0.39	0.392	-1.00

Genes that were down-regulated in the T-DNA line that were not complemented

Gene	Description	dNTF2	p-value	3HA	p-value	KK3 T-DNA
At4g23190	serine/threonine kinase - like protein (CRK11/AtRLK3)	-1.21	0.132	-0.71	0.150	-1.20

Genes that were up-regulated in the T-DNA line that were not complemented

Gene	Description	dNTF2	p-value	3HA	p-value	KK3 T-DNA
At3g19680	Expressed protein	**2.13**	0.001	**1.91**	1.1E-04	1.40
At2g21650	myb family transcription factor	**0.89**	0.022	**0.78**	0.012	0.80
At5g42040	26S proteasome non-ATPase regulatory subunit, putative	**0.52**	0.031	**0.89**	0.022	0.70

Genes that were up-regulated in the T-DNA line that were complemented differentially in the over-expression lines

Gene	Description	dNTF2	p-value	3HA	p-value	KK3 T-DNA
At4g38840	auxin-induced (IAA induced) protein, putative	0.28	0.321	**0.53**	0.011	0.80
At1g14210	ribonuclease T2 family	0.23	0.086	**0.82**	0.001	0.90

Genes that were up-regulated in the T-DNA line that showed WT expression levels in both over-expression lines

Gene	Description	dNTF2	p-value	3HA	p-value	KK3 T-DNA
At2g07020	protein kinase family - putative stress response	0.19	0.296	**0.49**	0.170	0.70

Four out of the 22 genes examined did not show any modification of gene expression profile upon expressing either *MKK3* variant, suggesting that these genes, At4g23190 (*CRK11*), At3g19680, At2g21650 and At5g42040 may not be involved in MKK3 signaling. Expression profiles of seven genes were restored to WT levels upon over-expression of either full-length *MKK3* or the *MKK3ΔNTF2* variant, while another seven genes that had been down-regulated in the SALK 051970 line relative to WT were now up-regulated upon over-expression of either full-length *MKK3* or the *MKK3ΔNTF2* variant. This elevated response may be a consequence of the ectopic expression of the *MKK3* variants in tissue compartments in which MKK3 is not normally expressed, or it could reflect a greater sensitivity of these particular genes to the specific level of MKK3 being generated in the transformed cells.

Four genes were complemented differentially in the two transgenic lines. The genes encoding the auxin-induced (IAA) protein (At4g38840) and At1g14210 were up-regulated in the SALK 051970 T-DNA insertion line and remained so in the full-length *MKK3* over-expression line, whereas in the *MKK3ΔNTF2* line, their expression returned to WT levels. In the case of the gene encoding NAP (At1g69490), expression of this gene was restored to WT levels in the *MKK3ΔNTF2* line, whereas its expression profile was reversed in the full-length-*MKK3* line. In the SALK 051970 T-DNA insertion line, this gene was >2-fold down-regulated relative to WT plants, but in the full-length *MKK3* over-expression line, it was now 3-fold up-regulated. Finally, a third case displayed the opposite pattern; the C2H2 zinc-finger transcription factor encoded by At5g04340 was two-fold down-regulated in the SALK051970 line relative to WT, but was expressed at WT levels in the full-length *MKK3* over-expression line and two-fold up-regulated in the *MKK3ΔNTF2* over-expression line.

The ability of either variant of MKK3 to consistently either restore or alter the gene expression profile for the majority of genes examined indicates that transcription of these genes is controlled, at least in part, by MKK3 signaling. However, it is important to recognize that all of these experiments examined plants that had been grown under normal conditions, and at this point I have no information concerning the activation status of MKK3 under such conditions. Therefore, while I assume that these modified expression profiles result from the relative abundance of MKK3 protein (full-length or truncated, as the case may be) in the plant, I cannot determine from these analyses whether it is the kinase-activated form of MKK3 that is responsible.

Other phenotypic characteristics of transgenic plants carrying CaMV *35S:MKK3* variant constructs

The relative levels of over-expression of the full-length *MKK3* and the *MKK3ΔNTF2* variant in the SALK 051970 background were 2.2 fold and 1.8 fold respectively. From the transcriptional profiling experiments it was clear the ectopic over-expression of both of these constructs has an impact on the status of the transcriptome of rosette tissue, suggesting that there may be phenotypic consequences of over-expression of these *MKK3* variants.

Initially, the general growth characteristics of each over-expression line were compared with growth of the SALK 051970 T-DNA insertional mutant line (Table 3.16). Because previous experiments had demonstrated that there were no fundamental differences between the SALK 051970 T-DNA insertional mutant line and WT (Table 3.2), the latter was not included in these experiments.

Table 3.16 Comparison of SALK 051970, CaMV *35S:MKK3* and CaMV *35S:MKK3ΔNTF2* plant growth on soil. Seeds were imbibed at 4°C in the dark for 48 hours prior to spreading on Redi-Earth®. Over a period of 50 days growth characteristics were observed and scored for differences between any of the lines. Any differences are described below.

Age/Trait	3	7	10	14	18	21	24	28	31	35	38	42	45	50
Time to germinate	=													
Time to first leaves		=												
petiole length of largest leaf set at bolting						=								
leaf size		=	=	=	=	=	=	=	=	=	=	=	=	=
leaf shape		=	=	=	=	=	=	=	=	=	=	=	=	=
leaf colour		=	=	=	=	=	=	=	=	=	=	=	=	=
leaf margins								=	=	=	=	=	=	=
stem length						=	=	=	=	=	=	=	=	=
stem colour						=	=	=	=	=	=	=	=	=
overall health in soil	=	=	=	=	=	=	=	=	=	=	=	=	=	=
time to bolting						=	=							
# leaves when bolting						=	=							
bolt length								=	=	=	=	+[1]	+[1]	+[1]
stem length								=	=	=	=	+[1]	+[1]	+[1]
time to flower							=							
# flowers per bolt							=	=	=	=	=	=	=	
flower morphology								=	=	=	=	=	=	
silique length									=	=	=	=	=	=
# of siliques								=	=	=	=	=	=	=
seed set (gross mass per plant)														=
speed of senescence										=	=	=	=	
leaf browning										=	=	=	=	=
stop flowering												=		

Equivalent (=)
+[1] Both 35S over-expression lines appeared to have slightly longer bolts/primary stems but the difference was not statistically significant ($\alpha<0.05$)

The majority of plants displayed no gross differences in growth patterns relative to the SALK 051970 T-DNA insertion line. However, at a rare frequency (~2%), severely growth-inhibited plants were observed for both the full-length *MKK3* and the *MKK3ΔNTF2* lines. These plants displayed small (~2 cm diameter) rosettes and very fine bolts that at maturity were an average of

18 cm in length. Furthermore, these plants appeared sterile and did not produce seeds. Due to the low frequency of appearance of this phenotype and the inability to obtain seeds from affected plants for further phenotypic analysis, experiments examining the nature of this phenotype were not pursued.

These plants were also subjected to a similar panel of abiotic and biotic treatments as was conducted for the SALK 051970 and WT plants to determine if there were treatment-specific phenotypic consequences of ectopic over-expression of the MKK3 variants (Table 3.17).

Table 3.17. Summary of treatment panel to identify phenotypic differences between the SALK 051970 T-DNA insertional mutant, *35S:MKK3* and *35S:MKK3ΔNTF2* lines.

Treatment	Concentration/Description	Result
Jasmonate (JA)	Germination on 1 μM JA	No difference in germination or continued growth
ACC (ethylene)	Germination on 2 μM ACC	No difference in germination or continued growth
ACC (ethylene)	Growth in the dark in presence of 2 μM ACC	No difference in growth
1-naphthylphthalamic acid (NPA)	Germination on 5 μM NPA	No difference in germination or continued growth
IAA (auxin)	Germination on 1 μM IAA	No difference in germination or continued growth
2,4-D (auxin)	Germination on 1 μM 2,4-D	No difference in germination or continued growth
ABA	Germination on 15 and 50 μM ABA	No germination of any line
Kinetin (cytokinin)	Germination on 0.5 μM kinetin	No difference in germination or continued growth
Gibberellin (GA)	Germination on 10 μM GA	No difference in germination or continued growth
Epi-brassinolide (BR)	Germination on 1 μM BR	No difference in germination or continued growth
Salicylic acid (SA)	Germination on 200 μM SA	No difference in germination or continued growth
SA	Transfer of 10-day old seedlings to 200 μM SA	No difference in growth
Cold	24 hours exposure to 4°C cold treatment	No difference in recovery
Cold	Continued growth at 4°C	No difference in growth
Desiccation	Stop watering bolting plants for a period of 5 days, followed by a return to a normal watering regime	No difference in recovery
NaCl	Germination on 50, 100, 150 and 200 mM NaCl	No difference in germination or continued growth
NaCl	10-day old seedlings transferred to100, 200 and 300 mM NaCl	No difference
Sucrose	Germination on 2% and 5% sucrose	No difference in germination or continued growth
Ozone	500 ppb for 8 hours followed by a 16 hour recovery period	No difference in ozone sensitivity
Potassium chloride	Germination on 50 mM KCl	No difference in germination or continued growth
Sorbitol	Germination on 5% sorbitol	No difference in germination or continued growth
LiCl	Germination on 15 mM LiCl	No difference in germination or continued growth
Caffeine	Germination on 2 mM caffeine	No difference in germination or continued growth
Pseudomonas syringae infection	21-day old rosette leaves were infiltrated with culture at either an OD_{600} of 0.001 (resistance dose) or 0.0001 (susceptibility dose) and plants were monitored for three days	No difference in susceptibility or resistance

Although several genes were mis-regulated in the *35S:MKK3* and *35S:MKK3ΔNTF2* lines, the ectopic over-expression of these constructs did not create any visible or conditional phenotypic differences within the panel of tests shown. This indicates that either over-expression of these

MKK3 variants is of no consequence to the plant, or that more subtle and/or treatment-specific phenotypes are involved that have yet to be detected.

Analysis of transgenic Arabidopsis plants expressing a dexamethasone-inducible *MKK3 RNAi* construct

To examine the effect of the elimination of MKK3 signaling from the plant by another method, a dexamethasone-inducible RNAi cassette targeted to the 5' region of the *MKK3* transcript was constructed and introduced into WT plants via the floral dip method. Twenty-five hygromycin B-resistant T1 plants were transferred to soil and allowed to grow until seed set. Induction of *MKK3* silencing could not be analysed in this generation due to the possibility that induction of gene-silencing might be lethal to the plant. T2 seeds from these plants were therefore screened on the basis of hygromycin B resistance. Preliminary tests of the function of the *MKK3 RNAi* construct were conducted using 10-day old seedlings that were submerged in a 10 μM dexamethasone solution for a period of ten minutes, following which the dexamethasone solution was aspirated. Complete plant tissue was harvested following a dexamethasone-induction period of 24 hours, and *MKK3* expression levels in the transgenic lines were quantified by quantitative real-time PCR. For this comparison, *MKK3* expression levels were measured in similarly treated *pTA7002* empty-vector lines (Figure 3.10).

Only partial suppression of MKK3 expression was observed in a number of lines, but six lines (Lines 3, 8, 9, 10, 14 and 16) with the strongest suppression were selected to carry through to the T3 generation.

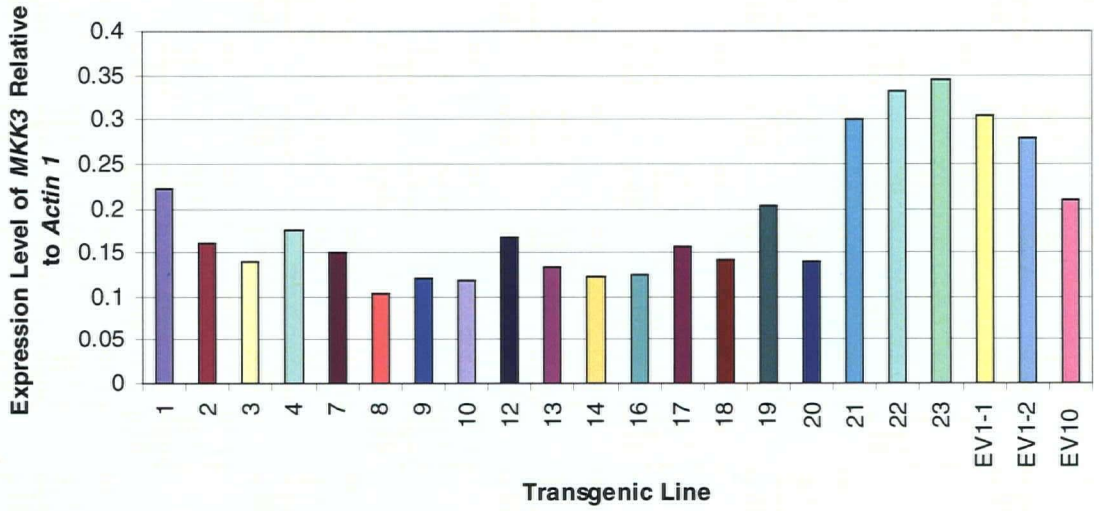

Figure 3.10. Quantitative real-time PCR analysis of 10-day old T2 seedlings expressing a dexamethasone-inducible MKK3-RNAi construct. Seedlings were treated by submersion in 10 μM dexamethasone for 10 minutes followed by aspiration of the dexamethasone solution. Induction of expression of the *MKK3 RNAi* construct was allowed to proceed for 24 hours. RNA was then extracted and gene expression quantified by real-time PCR analysis with MKK3QRT-F and MKK3QRT-R primers. Lines 3, 8, 9, 10, 14 and 16 were selected for analysis of T3 homozygotes.

Homozygous T3 plants were selected on the basis of 100% germination in the presence of hygromycin B, and the degree of *MKK3* silencing was re-examined in these lines by quantitative real-time PCR analysis (Figure 3.11). None of the RNAi lines showed any significant reduction in levels of MKK3 transcripts following dexamethasone induction. On the basis of these results, it was concluded that expression of this particular *MKK3 RNAi* construct failed to result in silencing of the *MKK3* message in the T3 generation.

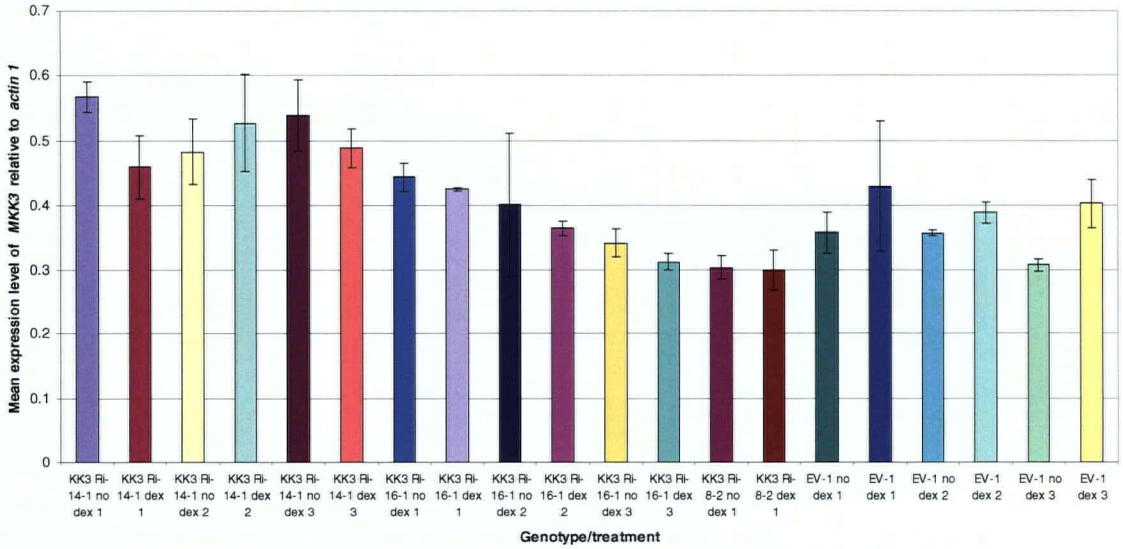

Figure 3.11. Real-time PCR analysis of MKK3 expression levels in 10-day old T3 homozygous seedlings expressing a dexamethasone-inducible *MKK3 RNAi* construct. Three biological replicates comprising cDNA derived from five individual plants were carried out for each of lines 14, 16 and EV1; a single replicate was analysed for line 8. Seedlings were either treated by submersion in a 10 μM dexamethasone (dex) or a mock-dexamethasone (no dex) solution for 10 minutes followed by aspiration of the dexamethasone solution. Induction of expression of the *MKK3 RNAi* construct was allowed to proceed for 24 hours, followed by RNA extraction and gene expression quantification by real-time PCR analysis with MKK3QRT-F and MKK3QRT-R primers.

To clarify whether the ability of the RNAi construct to silence *MKK3* might have been lost between the T2 and T3 generations, additional T2 and T3 plants were grown and dexamethasone-treated simultaneously, and the levels of *MKK3* expression were examined by quantitative real-time PCR (Figure 3.12). There was no evidence for silencing of MKK3 transcription in either generation, which suggests that the initial characterization of *MKK3* silencing in the T2 generation had been flawed.

Mock-treated control samples had not been included in my initial analysis, which meant that the most appropriate baseline for comparison between lines was not used. In a situation where strong suppression was being induced this might not have been an issue, but under circumstances where only subtle differences appeared between lines, the lack of an appropriate comparator

created the impression that significant RNAi silencing was occurring. This was clearly not the case.

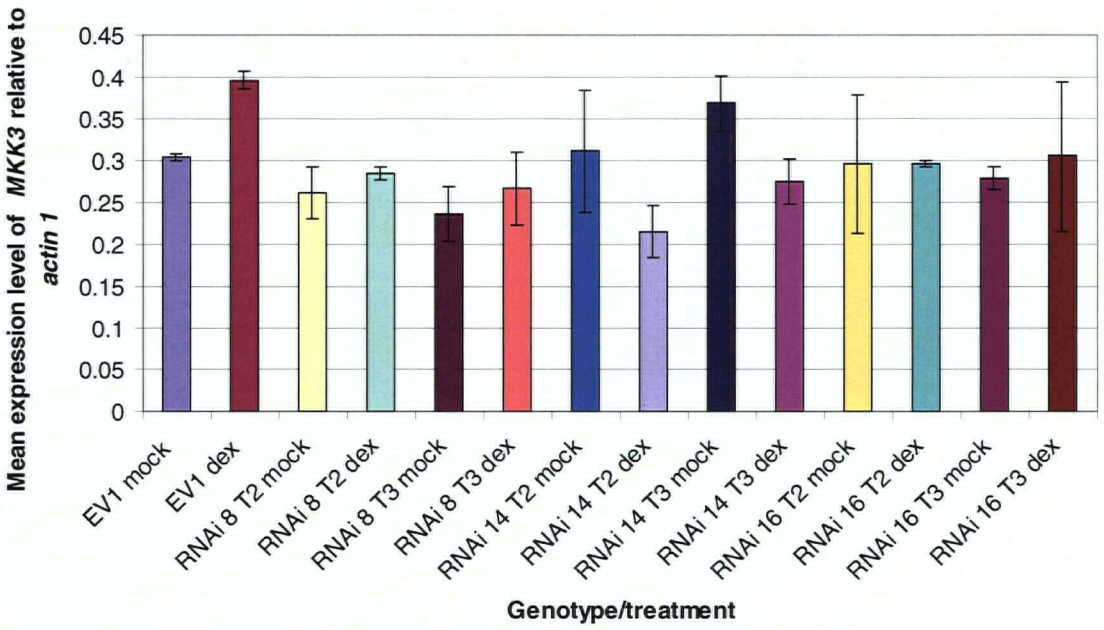

Figure 3.12. Real-time PCR analysis of MKK3 expression levels in 10-day old T2 heterozygous and T3 homozygous seedlings expressing a dexamethasone-inducible *MKK3 RNAi* construct. *MKK3* expression levels in cDNA derived from five individual plants for each line were quantified in T2 and T3 plants for each of lines EV1, 8, 14 and 16. In order to ensure all plants carried at least a single copy of the transgene, plants were cultivated on ½ MS plates supplemented with 35 μg/mL hygromycin B. Seedlings were either treated by submersion in a 10 μM dexamethasone (dex) or a mock-dexamethasone (no dex) solution for 10 minutes followed by aspiration of the dexamethasone solution. Induction of expression of the *MKK3 RNAi* construct was carried out for 24 hours, followed by RNA extraction and gene expression quantification by real-time PCR analysis with MKK3QRT-F and MKK3QRT –R primers. Treatment with dexamethasone has no significant effect on expression of *MKK3* in any generation or plant line tested.

DISCUSSION

Characterization of the SALK 051970 T-DNA insertion line

Characterization of genes using reverse genetics approaches relies heavily on two approaches: first, gene expression patterns can be studied using promoter-reporter constructs to learn where the gene product may be exerting its biological effects, and second, phenotypic analyses of over-expression and loss-of-function mutants often provides insight into those biological functions. Among all the Arabidopsis mutant lines available in nine publicly accessible collections (http://signal.salk.edu/cgi-bin/tdnaexpress) only the SALK 051970 T-DNA line contains an insertion into sequences encoding MKK3. Furthermore, analysis of TILLING mutant collections (Rodriguez and Huang, 2005) also did not reveal any point mutations in *MKK3* sequences.

The SALK 051970 line carries a T-DNA insert in exon 7 of the *MKK3* coding sequences (Figure 2.2), and RT-PCR analysis of plants homozygous for the T-DNA insertion using PCR primers designed to amplify the complete *MKK3* ORF showed that production of full-length *MKK3* transcripts was eliminated in this line (Figure 3.4). Thus, it appeared that the SALK 051970 line might represent an *mkk3-null* plant. However, additional PCR analyses using primers specific to the region upstream (5') of the T-DNA insert illustrated that truncated *MKK3* transcripts are still being actively produced in these plants (Figure 3.5). Because the PCR primers used to detect this truncated transcript were designed such that the forward and reverse primers bind to sequences in successive exons, I can conclude that these analyses detected spliced, aberrant *MKK3* transcripts and not genomic DNA or non-spliced mRNA.

The binary vector *pROK2* had been used to create the SALK T-DNA insertion lines and DNA sequencing of the insertion site has been carried out using PCR primers specific to the left border

region of the T-DNA insert (Alonso et al., 2003). Translations of the left border DNA sequence in all three reading frames indicate that a stop codon is encoded within no more than 90 base pairs, or 30 amino acids, in each frame (Figure 3.13). The T-DNA insert in SALK 051970 lies between sequences encoding the dual-specificity kinase domain and the NTF2 domain. Thus, if this aberrant mRNA were to be successfully translated, a truncated MKK3 protein containing only the dual-specificity kinase domain would be produced. In this case, the SALK 051970 line would represent a *mkk3Δntf2* plant rather than an *mkk3*-null plant. Immunoanalysis to try to resolve this question was unsuccessful, likely because of low antigenicity of the MKK3 peptides and/or the low abundance of MKK3 protein in the plant.

DNA sequence of left border region of *pROK2*

TATATTGTGGTGTAAACAAATTGACGCTTAGACAACTTAATAACACATTGCGGACGTTTTTAATGTACT
GGGGTGGTTTTTCTTTTCACCAGTGAGACGGGCAACAGCTGATTGCCCTTCACCGCCTGGCCCTGAGAG
AGTTGCAGCAAGCGGTCCACGCTGGTTTGCCCCAGCAGGCGA

Translation of left border sequence up to first stop codon

5'3' Frame 1

Y I V V Stop

5'3' Frame 2

I L W C K Q I D A Stop

5'3' Frame 3

Y C G V N K L T L R Q L N N T L R T F L Met Y W G G F S F H Q Stop

Figure 3.13. DNA sequence and three-frame translations of the left border region of *pROK2*. T-DNA insertion points in the SALK lines were identified by DNA sequence analysis using primers specific to the left border region of the T-DNA insert. The DNA sequence of the left border region immediately flanking the insertion site was translated to identify the first stop codon encoded in each reading frame. A stop codon is encoded within 90 base pairs in each reading frame, suggesting that the mRNA produced in the SALK 051970 T-DNA insertion line would include a premature stop codon immediately downstream of the insertion point.

Several mechanisms of ensuring RNA quality control are known to operate in eukaryotic organisms, including plants (Gonzalez et al., 2001; Isshiki et al., 2001; Fasken and Corbett, 2005; Hori and Watanabe, 2005). These include co-transcriptional quality control, in which mis-transcribed RNAs are subject to degradation, selectivity during mRNA export from the nucleus where improperly spliced mRNAs will not associate with export machinery, and translational quality control via nonsense-mediated mRNA degradation (NMD; Maquat and Carmichael, 2001). NMD is a process that recognizes aberrant transcripts containing premature stop codons and targets them for degradation via several different mechanisms (Fasken and Corbett, 2005). The existence of multiple mRNA quality control mechanisms might suggest that a protein will not be produced from the aberrant mRNA detected in the SALK 051970 T-DNA insertion line. However, my ability to detect a spliced form of the aberrant transcript indicates that this transcript was at least able to avoid degradation by co-transcriptional and, possibly, export quality control systems. It is interesting to speculate that perhaps the presence of coding sequence for a complete dual-specificity kinase domain with substantial homology to other Arabidopsis MAPKKs enabled the aberrant mRNA to resemble normal MAPKK transcripts sufficiently to be able to pass the quality screens.

The most likely form of detection of aberrant *MKK3* mRNA would therefore be NMD, where aberrant mRNA is degraded at the time of attempted translation initiation. The literature indicates that while aberrant mRNAs do result from T-DNA insertions, occasionally at WT levels, protein synthesis from these transcripts either is rare or cannot be detected (Sanderfoot et al., 2001; Ullah et al., 2001; Puizina et al., 2004). It is therefore certainly possible, and perhaps even probable, that RNA quality control mechanisms will prevent the production of a truncated

MKK3 protein in the SALK 051970 line. Nevertheless, without direct examination of MKK3 protein levels, I cannot confirm that the SALK 051970 line is truly a *mkk3-null* mutant.

Phenotypic analysis of SALK 051970 plants

The transient pattern of *MKK3* expression in developing floral tissues at sites that correlate with local auxin production (Chapter 2) suggested that MKK3 signaling may be involved in floral development, but no differences in floral organ appearance were observed in the SALK 051970 flowers. In addition, persistent expression of *MKK3* was observed in seeds that fail to develop completely (Chapter 2), but while this might be indicative of a function for MKK3 signaling in seed development, no differences in seed production were detected, as determined by gross seed weight per plant.

Because *MKK3* expression is concentrated in the meristematic region of the root tip, it was thought that elimination of MKK3 signaling might impair root development. However, when SALK 051970 T-DNA insertion and WT seeds were compared for germination rate and initial growth characteristics on ½ MS agar plates, no phenotypic differences were observed, either in germination rates, growth to 14-days-old or gross morphology as assessed using a dissecting microscope.

MKK3 promoter sequences are responsive to the phytohormones auxin and ABA and also to osmotic stress and heat shock (Chapter 2). Seedlings exposed to all of these treatments respond by displaying inhibition of root elongation. Untreated plants do not express *MKK3* in the zone of elongation of the root. However, *MKK3* expression is up-regulated in this region following exposure to root elongation-inhibiting treatments, suggesting a possible role of MKK3 signaling in preventing root growth. Therefore, if this hypothesis is correct, and if the SALK 051970 plants truly lack MKK3 functionality, I would have predicted that exposure of the T-DNA

insertion plants to auxin, ABA, osmotic stress or heat shock would not lead to inhibition of root growth. However, no differences were observed between SALK 051970 and WT plants germinated in the presence of 1 μM IAA (auxin), 1 μM 2,4-D (auxin), 10 or 50 μM ABA or 50-200 mM NaCl with respect to germination rate or subsequent growth in the presence of the respective media additive. In addition, 10-day-old SALK 051970 and WT seedlings transferred to 1 μM IAA, 50 μM ABA or 100-300 mM NaCl showed no phenotypic differences during the subsequent 10 days of growth on these media. I therefore conclude that either my model for the function of MKK3 is incorrect, or that the T-DNA insertion in the SALK 051970 plants does not disrupt MKK3 function, or that there is sufficient redundancy in MAPKK function in Arabidopsis to compensate for loss of MKK3 with respect to the phenotypic analyses conducted in this study.

I feel that the most likely explanation is the existence of extensive functional redundancy amongst MAPKKs, especially those such as MKK3 that appear to be linked to phytohormone signaling. It has often been observed that single loss-of-function mutants in various auxin-associated genes do not produce abnormal plant phenotypes due to functional redundancy (Bouche and Bouchez, 2001). Instead, loss-of-function phenotypes can then only be detected when combinatorial loss-of-function plants are studied (Bouche and Bouchez, 2001). This phenomenon is also common amongst ABA-associated gene families where *abi5*, *abf1* and *afb3* single loss-of-function mutants show minimal phenotypic effects in response to ABA (Finkelstein et al., 2005). However, *abi5/abf3* double mutants display enhanced germination in the presence of ABA, NaCl and sorbitol (Finkelstein et al., 2005).

Functional redundancy has also been observed amongst plant MAPKKs, where the MKK1/MKK2 and MKK4/ MKK5 pairs show at least partially overlapping function (Asai et al.,

2002; Teige et al., 2004). Phylogenetic analysis of Arabidopsis MAPKKs indicates that MKK3 is the only group B MAPKK in Arabidopsis (Ichimura et al., 2002; Hamel et al., 2006), which would argue against fully redundant functionality between MKK3 and other MAPKKs. The presence of the C-terminal NTF2 domain in MKK3 does not influence this conclusion, since a phylogenetic tree based upon multiple sequence alignments of only the protein kinase domains of each Arabidopsis MAPKK (Figure 3.14) also indicates that MKK3 is phylogenetically unique, which could be interpreted to mean that it is likely to be functionally distinct as well. On the other hand, the presence of a single NTF2 domain-containing MAPKK gene in the *Chlamydomonas* genome implies that a group B MAPKK similar to AtMKK3 may have been the predecessor of all plant MAPKKs. If this were the case, it is not out of the question that one or more of the nine additional MAPKKs encoded in the Arabidopsis genome could have retained the ability to provide MKK3 functions. If MKK3 functionality in the SALK 051970 T-DNA insertion line is truly lost, the lack of any clear phenotype may be a consequence of such redundancy. However, at this point, the possibility that MAPKK redundancy may be affecting the phenotype of the SALK 051970 plants must remain an interesting speculation.

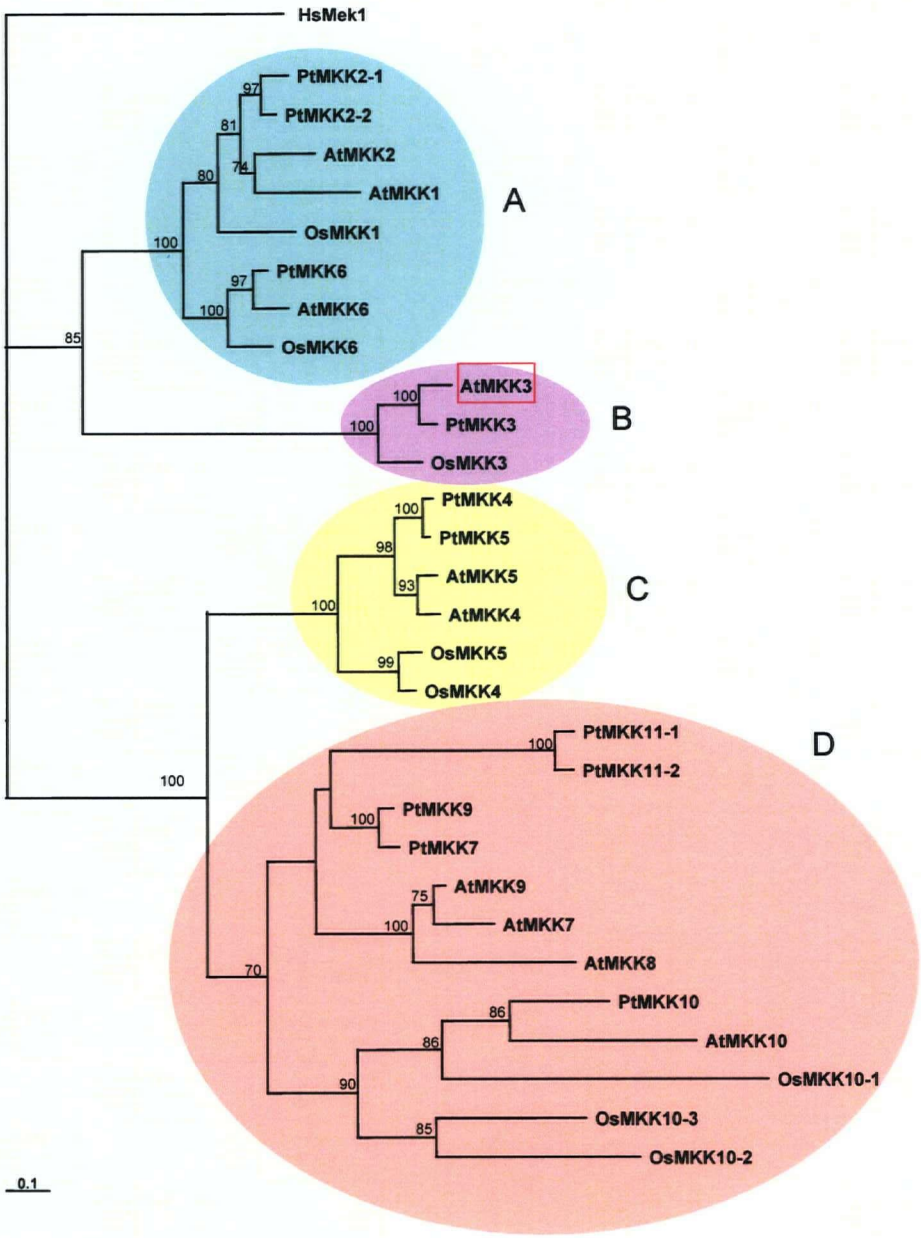

Figure 3.14. Phylogenetic analysis of MAPKK kinase domain sequences from Arabidopsis, rice and poplar. Adapted from (Hamel et al., 2006). Alignments were performed as described, using ClustalW and the phylogenetic tree was generated using TreeView. While AtMKK3 and group B MAPKKs are most closely related to group A MAPKKs, they appear to be phylogenetically unique.

It is also possible that an abnormal, discrete phenotype was simply not detected by my analyses.

For example, *MKK3* expression appears to be closely linked to the sites of auxin biosynthesis in

floral tissues (Chapter 2). Although no macroscopic defects were detected in floral tissues, higher resolution methods such as analysis by scanning electron microscopy might be required to detect more subtle cellular defects. Ultimately, it must be borne in mind that we have no knowledge of the biological events that result in the phosphorylation of MKK3 *in vivo*; i.e. in activation of MKK3 signaling. It is possible that MKK3 is involved in highly specialized signaling events, and thus under most circumstances the plant can develop normally in the absence of MKK3. This is not an uncommon phenomenon for MAPK signaling; *S. cerevisiae* mutants lacking the complete complement of MAPKs are viable as long as these strains are cultured under optimal conditions (Madhani and Fink, 1998).

It should also be recognized that activation of MAPK signaling modules is not always required in order for them to execute their biological functions. MAPK signaling components, including MAPKKs, are known to have homeostatic functions in the absence of activating stimuli. For example, human MEK1/2 forms a heterodimeric MEK1/2-ERK1/2 cytosolic complex that prevents nuclear localization of ERK1/2 in the inactive form (Kondoh et al., 2005). Other MAPK scaffolds have been suggested to prevent unregulated activation of signaling pathways (Chong et al., 2003). Of particular interest is the yeast MAPKK, Pbs2. Pbs2 acts as a scaffold protein for the MAPKKK, Ste11 and the downstream MAPK, Hog1, and it functions as a MAPKK involved in the osmolarity response (Posas and Saito, 1997). However, Ste11 is also involved in the mating response and pbs2-null strains show increased crosstalk between the osmolarity and mating response signaling pathways (O'Rourke and Herskowitz, 1998). This is thought to be due to due to excess free Ste11 which results from the deficiency of Pbs2 scaffold (O'Rourke and Herskowitz, 1998).

Construction of MKK3-RNAi silenced lines

Due to the ambiguity surrounding the SALK 051970 line, I used an alternative approach to generate MKK3 loss-of-function mutants; i.e. an RNAi construct targeted to the N-terminal region of *MKK3*. I decided against using an RNAi sequence targeted to the 3' region of the *MKK3* transcript, since this region contains sequences encoding the NTF2 domain of MKK3. The deletion of NTF2 in yeast is lethal, and the sequences encoding the NTF2 domain of MKK3 are similar to sequences encoding discrete NTF2 genes in Arabidopsis. Targeting this region of MKK3 thus had the potential to silence a possibly essential gene.

The RNAi construct was placed under the control of a dexamethasone-inducible promoter system (Aoyama and Chua, 1997) in order to allow for specific and controlled induction of *MKK3* silencing. Although twenty-five transformed lines were recovered on the basis of antibiotic selection, I was ultimately unable to detect induced gene silencing in any of the lines tested (Figure 3.13).

The most plausible explanation for this may be the short target region included in the *MKK3 RNAi* construct. In order to avoid silencing other MAPKK genes, I had selected as my target a 211bp region of the 5' terminus of the MKK3 transcript, but RNAi-based gene silencing in Arabidopsis commonly uses RNAi constructs that include 400-800 base pairs of target gene sequence (Wesley et al., 2001; Watson et al., 2005). It is not clear how many of the various 21-22 base pair RNA molecules ultimately derived from the large hairpin RNA (hpRNA) during processing of RNAi transcripts are capable of inducing silencing of the target gene, but presumably not all hpRNAs will be functional. Thus, while using a smaller RNAi cassette, as I did, reduces the possibility of triggering non-specific gene silencing, it also may diminish the chance for efficient silencing of the targeted gene.

It is, of course, also possible that I was unable to induce stable silencing of MKK3 because the plant is unable to tolerate manipulation of MKK3 outside of a narrow window of expression levels. This issue is discussed in more detail later.

Characterization of MKK3 over-expression plants

To explore the possibility that simply increasing the intracellular concentration of MKK3 might be sufficient to perturb cellular functions in a manner that would provide insight into its biological function, I generated transgenic lines of Arabidopsis over-expressing either full-length *MKK3* or an *MKK3ΔNTF2* variant. These constructs were driven by the CaMV 35S promoter and were expressed in the SALK 051970 T-DNA insertion line background. My goal was to characterize the visible and conditional phenotype of both types of plants, including the ability of the two constructs to complement the changes in expression of the genes that appeared to be perturbed in the SALK 051970 line (see section Function of the NTF2 domain)

Despite analyzing thirty-five independent hygromycin-resistant T1 plants transformed with each *MKK3* over-expression construct (full-length *MKK3* and *MKK3ΔNTF2*), the highest level of over-expression I could detect for either construct was approximately two-fold. The highest expressing lines of each *MKK3* variant were indistinguishable from WT plants, both in response to external stimuli and throughout development. Given that native *MKK3* is expressed at relatively low levels, and that the CaMV 35S promoter typically directs over-expression of transgenes at levels much higher than the two-fold increase observed in these experiments (Ow et al., 1987; Lam et al., 1989; Jackson et al., 2002; Zabala et al., 2005; Sridha and Wu, 2006), I had anticipated that I would recover transgenic plants with much greater over-expression levels of *MKK3*.

There are several possible explanations for my inability to recover such high expressing lines. First, high-level over-expression of transgenes is capable of inducing post-transcriptional gene silencing (PTGS; Baulcombe, 2004), so perhaps over-expression of *MKK3* initiates this process. Alternatively, recent studies have illustrated that control of gene expression by miRNAs is widespread in plants, and is particularly common amongst developmental, auxin- and stress-associated genes (Sunkar and Zhu, 2004; Carrington, 2005). Although no genes encoding protein kinases have been reported to be targets of miRNAs in plants, it is clear that that miRNAs target many different types of genes, including those encoding transcription factors, metabolic enzymes (superoxide dismutase, dehydrogenases), transporters and receptors (Millar and Waterhouse, 2005). Furthermore, two miRNA prediction algorithms, FindMiRNA (http://sundarlab.ucdavis.edu/mirna/) and Web MicroRNA designer (http://wmd.weigelworld.org/bin/mirnatools.pl), predict the presence of multiple putative miRNA target sequences within *MKK3* transcripts.

A third possible reason why transgenic plants highly over-expressing either variant of *MKK3* were not recovered could be lethality of *MKK3* over-expression. My analysis of *MKK3 promoter:GUS* reporter plants revealed that *MKK3* expression does not occur in properly developed seeds, but could be clearly detected in seeds that failed to develop normally (Figure 2.7). The ovule is the target of transformation of Arabidopsis by *Agrobacterium tumefaciens,* and expression of T-DNA-borne transgenes can be detected throughout seed development (Ye et al., 1999). Perhaps constitutive expression of *MKK3* at high levels impairs seed development, preventing the recovery of highly expressing lines. This would be consistent with a model in which MKK3 functions as a negative regulator of development.

Transcriptional profiling of SALK 051970 and MKK3 over-expression plants

Microarray analysis of gene expression patterns in the SALK 051970 T-DNA line, comparing it with WT plants, successfully identified a small number of genes whose expression was being affected by the T-DNA insertion into the *MKK3* locus, in the absence of externally applied stimuli. This limited difference could be the result of using untreated plants as the source tissue for expression profiling. My *MKK3 promoter:GUS* reporter studies had indicated that MKK3 signaling is involved in some phytohormone and stress responses (Chapter 2), so it can probably be assumed that not all aspects of MKK3 signaling were active in the microarray experiments. The differentially expressed genes that were identified could be indicative of signaling defects leading to an altered, but stable, developmental state, in which case the detected genes might represent pleiotropic effects associated with this new state. Alternatively, they could indicate that, in the absence of fully functional MKK3, the plant does not possess the full complement of proteins capable of directing appropriate responses to the usual MKK3-activating stimuli. As a result, signaling initiated upstream of MKK3 might generate pathway crosstalk once it encounters a block or bottleneck at the MKK3 level. However, both of these scenarios would imply that the transcriptional differentials detected are a reflection of aberrant signaling. This is not consistent with the pattern of genes affected in the microarray analyses, which appears to correlate rather well with the data from the *MKK3 promoter:GUS* reporter studies, insofar as both approaches link MKK3 signaling to development, phytohormone- and stress-responses in Arabidopsis.

Up-regulated genes in the SALK 051970 line

GO reports for up-regulated genes in the SALK 051970 T-DNA gene lists suggested that several genes are associated with stress responses and further that several of these genes encode

enzymes such as protein kinases, hydrolases and transferases, examples of which have been linked to stress signaling. Further analysis of the GO terms assigned to these genes indicated that three genes were reported as being auxin responsive, At1g72430, At2g21220 and At4g38840. Each of these genes belongs to the SAUR gene family, of which there are 72 members in Arabidopsis (Hagen and Guilfoyle, 2002). Three classes of genes are known to be involved in the early response to auxin treatment, the Aux/IAAs, GH3s and SAURs (Knauss et al., 2003). Expression of SAUR genes is typically up-regulated within minutes of auxin exposure, although little else is known regarding the function of the SAURs (Hagen and Guilfoyle, 2002). It has been suggested that they are involved in auxin signal transduction, perhaps in controlling the effects of downstream genes, and in some cases expression of SAURs appears be specific to elongating tissues (Hagen and Guilfoyle, 2002; Knauss et al., 2003). Furthermore, the *Zea mays* ZmSAUR2 can bind to calmodulin, suggesting that this SAUR at least may help mediate cross-talk between calcium signaling networks and the auxin response (Yang and Poovaiah, 2000). Although no SAUR over-expression experiments have been reported, *dst* mutants that result in up-regulation of at least one Arabidopsis SAUR, (*SAUR AC1*) display no visible phenotypes (Perez-Amador et al., 2001). This is consistent with *SAUR* up-regulation as a consequence of T-DNA insertion into *MKK3* coding sequences.

Two genes encoding proteins with peptidase activity were also identified by these analyses, one of which (At5g42040) encodes a regulatory element of the 26S proteasome regulatory complex (www.tair.org). The proteasome has been linked to several hormone signaling pathways including auxin and ethylene (Liu and Zhang, 2004; Dharmasiri et al., 2005) further suggesting a role of MKK3 signaling in auxin signaling. However, because the expression of this gene may not have been affected by re-expression of either MKK3 variant in the SALK 051970 T-DNA

insertional mutant (expression of At5g42040 in the *MKK3ΔNTF2* over-expression line appeared similar to WT levels but remained statistically higher (~0.5 fold)) it is not certain that this protein is associated with MKK3 signaling.

An expansin (At4g38400/AtEXLA2) was also up-regulated in the SALK 051970 line. In Arabidopsis, expansin genes form a 35-member gene family and the encoded proteins are thought to function in promoting cell growth by loosening cell walls and allowing for cellular expansion (Sampedro and Cosgrove, 2005). Although AtEXLA2 has not been specifically studied, examination of expansin over-expression and loss-of-function plants typically show enhanced and inhibited growth respectively (Sampedro and Cosgrove, 2005). Up-regulation in the SALK line of an enzyme promoting cell growth is consistent with the model of MKK3 inhibiting cell growth, as suggested by *MKK3 promoter:GUS* reporter experiments (Chapter 2).

Down-regulated genes in the SALK 051970 line

The majority of differentially regulated genes in the SALK 051970 line were down-regulated, which is consistent with the hypothesis that these plants are not fully equipped for the response to MKK3-activating stimuli. GO reports for the down-regulated genes illustrate a possible link to stress signaling, since 15% of the down-regulated genes were annotated as stress, abiotic or biotic stimulus-induced. Identification of over-represented GO terms amongst the down-regulated genes revealed over-representation of multiple GO terms including developmental, drought-, ABA- and sugar-responsive genes.

Down-regulated genes encoding transcription factors

GO annotation analyses also indicated that 16% of the down-regulated genes encoded transcription factors. Examination of the promoter regions of each of these genes indicated that

several stress- and hormone responsive CAREs were over-represented (Table 3.13). Of particular interest were the W-box, the ABRE motif, Myb binding sites and the CARGCW8GAT motif.

Myb transcription factors are involved in development and environmental stress responses in plants (Yanhui et al., 2006). The MYB4 binding domain was found in the promoter of *MKK3* itself, in the promoters of 90% of the down-regulated transcription factor genes and in the promoters of 82% of all the down-regulated genes. Another Myb binding domain, MYB1AT, was statistically over-represented in *MKK3* and the differentially regulated genes, suggesting that *MKK3* and MKK3-related gene expression can be influenced by stress and by development-related Myb transcription factors.

The CARGCW8GAT motif is the binding site of the MADS box transcription factor, AGAMOUS (Tang and Perry, 2003). Slight variations of this site are also commonly bound by several other MADS box transcription factors. These collectively comprise a large family of transcription factors generally associated with floral, root and fruit development (Becker and Theissen, 2003; Tang and Perry, 2003), all of which are possible areas of function of MKK3 signaling. Although no MADS domain transcription factors were differentially expressed in the SALK 051970 T-DNA insertion line, over 80% of the promoters of the differentially expressed genes contained the CARGCW8GAT motif, suggesting that many of these genes are developmentally regulated. That the *MKK3* promoter itself does not contain this motif might suggest that post-translational activation of MKK3 signaling is needed for up- or down-regulation of these development-associated genes.

The W-box is recognized and bound by WRKY transcription factors, and this motif is found in the promoters of genes involved defense and abiotic stress (Eulgem et al., 2000; Ulker and

Somssich, 2004). Four genes encoding WRKYs were down-regulated in the SALK 051970 T-DNA insertion line - WRKY11, 25, 33 and 40. The expression patterns of three of these, *WRKY11, WRKY33,* and *WRKY40* were analysed in the *MKK3* variant over-expression lines. Both *WRKY11* and *WRKY33* became up-regulated upon over-expression of either full length *MKK3* or *MKK3ΔNTF2*. It appeared as though the same expression pattern might existed for *WRKY40* in both lines, but due to insignificant statistical analyses I could only conclude that over-expression of *MKK3* and *MKK3ΔNTF2* in the SALK 051970 T-DNA insertion line would restore *WRKY40* expression to WT levels. Nonetheless, a link between MKK3 and WRKY transcription factors is clear.

Several of the differentially regulated genes in the SALK 051970 T-DNA insertion line contained multiple W-boxes in their promoters, further suggesting that MKK3 signaling is associated with WRKYs and their targets. A link between MAPK signaling and WRKYs has previously been reported. WRKY22 and WRKY29 were found to be downstream components of a MAPK signaling module involving MEKK1-MKK4/5-MPK3/6 (Asai et al., 2002).

The ABRE motif (PyACGTGGC), and similar derivatives such as the G-box (CACGTG) and coupling element (CGCGTG), are consistently found in the promoters of ABA-inducible genes (Fujita et al., 2005). This consensus is recognized and bound by leucine-zipper, bZIP transcription factors such as AtHB7 (At2g46680) that themselves are regulated by ABA and may be integral components of ABA-induced signaling pathways. In addition to the ABRE motif being over-represented in the promoters of the down-regulated transcription factors in my gene list, it, along with the similar CACGTG motif, was also over-represented amongst all the down-regulated genes (Table 3.6), again suggesting an association of MKK3 with ABA-controlled gene expression.

Assuming that decreased expression of genes encoding transcription factors results in decreased steady-state levels of the corresponding proteins, and the relative abundance of transcription factors in the down-regulated gene list, one might have anticipated finding a greater number of mis-regulated genes in the SALK 051970 T-DNA insertion lines. However, many transcription factors exist in both inactive and active forms that can be interconverted by post-translational modifications such as phosphorylation. MAPKs normally phosphorylate their protein substrates on serine or threonine residues that are followed by a proline residue (Widmann et al., 1999; Roux and Blenis, 2004). When I examined the amino acid sequences of the differentially regulated transcription factors, I was able to identify possible -S/T-P- motif MAPK phosphorylation sites in each of the transcription factors, with the exception of At3g15500. However, if the more stringent phosphorylation motif -P-X-S/T-P- is used, as described by Widmann et al (1999), only two transcription factors, At4g37180 and At5g47230 (*ERF5),* would appear to be possible MAPK substrates.

On the other hand, these predictions are not always reliable, since the Arabidopsis metabolic enzyme, ACS6, was recently found to be phosphorylated on multiple serine residues by the MAPK, AtMPK6, and although all serine residues phosphorylated in this protein are followed by a proline, not all correspond to the more stringent P-X-S/T-P MAPK target sequence (Liu and Zhang, 2004). Thus, any future analyses of the transcription factors identified in the microarray analysis for their potential to serve as MAPK substrates should probably include the complete set of 23 transcription factors.

Both At4g37180 and *ERF5* show overlapping expression patterns and are expressed at similar levels to *MKK3* (Genevestigator). At4g37180 encodes a Myb-family transcription factor of

unknown function, although the expression of this gene is up-regulated in response to osmotic stress and ABA (Genevestigator).

ERF5 is a transcriptional activator of genes containing GCC boxes (AGCCGCC) in their promoter sequences (Fujimoto et al., 2000) and *ERF5* expression is induced by ethylene, salt and cold stress (Genevestigator). While no other studies of ERF5 have been reported, other ERFs have been found to function in several aspects of the plant stress and hormone responses, including ethylene, ABA, salt and jasmonate signaling (Fujimoto et al., 2000; Lorenzo et al., 2003; Fischer and Droge-Laser, 2004; Zhang et al., 2004; Yang et al., 2005). Three other ERF-type transcription factors were down-regulated in the SALK 051970 T-DNA insertion line - *ERF4* (At3g15210), *ERF8* (At1g53170) and *ERF11* (At1g28370). Each of these potentially functions in opposition to ERF5, in that they are reported to act as transcriptional repressors (Yang et al., 2005), but they may be functionally related since they all appear to be expressed in response to common stimuli (Genevestigator). Over-expression of *ERF4* (approximately 10-fold) conferred ethylene- and ABA-insensitive and NaCl-hypersensitive phenotypes (Yang et al., 2005). In the *35S:MKK3* over-expression plants constructed in the SALK 051970 T-DNA background, *ERF4* expression was slightly down-regulated (2-fold, $p<0.05$). The abnormal phenotypes resulting from *ERF4* over-expression could suggest that decreased *ERF4* expression in the SALK 051970 T-DNA insertion line would result in inappropriate responses to ethylene, ABA or NaCl but such a phenotype was not observed in the SALK line. This could simply reflect extensive functional redundancy amongst ERFs, since they form a large gene family of >100 genes (Fujimoto et al., 2000). It could also be due to decreased *ERF4* expression without significant reduction in ERF4 protein levels. Recently, it was reported that *erf4-1* loss-of-function mutants do not display abnormal phenotypes when grown under optimal conditions, but

that they are less susceptible to *F. oxysporum* infection and are more sensitive to JA-induced root growth inhibition (McGrath et al., 2005). It would be interesting to examine the expression pattern of *MKK3* in the *ERF4* over-expression and loss-of-function lines to determine if there is reciprocal control of gene expression, or if MKK3 signaling appears to contribute to the observed hormone-sensitivity phenotypes.

When the expression of *ERF8* and *ERF11* was examined in the *MKK3* variant over-expression lines, it was clear that MKK3 is associated with the expression of both of these genes, since *ERF8* expression was restored to WT levels and *ERF11* expression became approximately four-fold up-regulated in both *35S:MKK3* and *35S:MKK3ΔNTF2* lines.

Another family of transcription factors that was mis-regulated in the SALK T-DNA insertion line was the NAM family, of which six members were down-regulated (Table 3.21). While none of these transcription factors contained the stringent MAPK substrate motif, all did contain serine or threonine residues immediately upstream of a proline and are thus possible substrates of MAPKs. NAM transcription factors belong to the 105-member NAC super-family of development-associated transcription factors characterized by a N-terminal NAC domain (Ooka et al., 2003). Other NAC domain transcription factors have been found to be involved in plant development, with *CUP-SHAPED COTYLEDON (CUC)* and *NAC1* being involved in floral/shoot apical meristem and lateral root development. In addition, *NAC1* expression is induced by exogenous auxin and appears to be a mediator of auxin signaling (Xie et al., 2000). A recent large-scale classification of NAC-family genes assigns the following systematic names to the down-regulated NAMs in the SALK 051970 T-DNA insertion line: ANAC87 (At5g18270), ANAC55 (At3g15500), ANAC32 (At1g77450), ANAC102 (At5g63790), ANAC29 (At1g69490), and ANAC72 (At4g27410; Ooka et al., 2003). Although each of these

had been annotated as a NAM-family member using GO annotation, the analysis by Ooka et al (2003) reported that, while all of these are true NAC-family members, only ANAC87 is a NAM subgroup transcription factor. ANAC55 and ANAC72 are AtNAC3 NACs, ANAC32 and ANAC102 are ATAF NACs, and ANAC29 is a NAP group member (Ooka et al., 2003). Interestingly, NAM-group members are believed to play developmental roles, while AtNAC3, NAP and ATAF members appear to be involved in stress responses (Ooka et al., 2003). The link between MKK3 and two of these genes, *ANAC29* and *ANAC72*, was substantiated in the *MKK3* variant over-expression lines; *ANAC72* expression was restored to WT levels upon over-expression of both full length *MKK3* and an *MKK3ΔNTF2* variant in the SALK 051970 T-DNA insertion background, whereas the expression of *ANAC29* was complemented differently in each line. It was restored to WT levels in the *MKK3ΔNTF2* over-expression line, while it was up-regulated (~4 fold) in the full length *MKK3* over-expression line. This suggests that, while *ANAC29* expression is influenced by the kinase domain, additional control over *ANAC29* expression is influenced by the NTF2 domain of MKK3.

Phenotypic characterization of loss-of-function mutants for MKK3-associated genes might help clarify the role of MKK3 in the stress-response, but since each of these transcription factors are components of very large gene families, the chance for functional redundancy for any given transcription factor seems high. Perhaps evaluation of over-expression lines for each of these transcription factors will prove more insightful. Such an effort is underway, at least commercially, by Mendel Biotechnology (http://www.mendelbio.com/index.html).

Differentially-regulated auxin-associated genes

Analysis of the differentially regulated genes in the SALK 051970 T-DNA line that encode transcription factors indicated that it is likely that MKK3 signaling, or perhaps MKK3 in the

inactive state, is associated with plant stress responses, plant development, or perhaps both. To explore this idea further, the complete list of differentially regulated genes was analysed for genes known to be involved in stress-related phytohormone responses and development. GO terms over-represented in the lists of differentially regulated genes suggested that seven genes, At4g27410 (ANAC72/RD26), At1g69640 (ANAC29/NAP), At3g15500 (ANAC55/NAC3), At5g18720 (ANAC87), At1g77450 (ANAC32), At4g38400 (AtEXLA2) and At4g35770 (SEN1) were involved in development. Each of these genes have been described in previous sections, with At4g27410, At1g69640, At3g15500, At5g18720 and At1g77450 encoding down-regulated NAC family transcription factors and At4g38400 and At4g35770 being auxin-responsive genes. This overlap of stress-, hormone- and developmentally-induced genes highlights the range of cross-talk between these pathways and illustrates the need for more detailed characterization of the function of each specific gene. Nonetheless, promoter analyses of each of these genes, including *MKK3* indicated significant overlap of CAREs (Table 3.12) suggesting that these genes are co-regulated, and are thus likely involved in common biochemical pathways.

Similar analyses were performed to identify auxin-responsive genes. Because of the developmental roles of auxin (Casimiro et al., 2003; Jenik and Barton, 2005; Leyser, 2005; Woodward and Bartel, 2005; Aloni et al., 2006) I expected there would be significant overlap in the auxin- and development-associated gene lists. Eight genes were identified, which included the three previously discussed up-regulated SAURs (At1g72430, At4g38840, and At2g21220). The remaining genes were all down-regulated; one encodes a PINOID binding protein (PBP1; At5g54490), another encodes an auxin efflux carrier protein, PIN7 (At5g15100), the fourth encodes the ethylene biosynthetic enzyme, ACS6 (At4g11280), and the last two encode an auxin

biosynthetic enzyme (At4g27260) and an auxin response transcription factor (ARF9; At4g23980).

Although not a substrate for pinoid kinase (PID), PBP1 was shown to interact with this kinase in a calcium-dependent fashion. PID is thought to promote polar auxin transport and PBP1 may function upstream of PID to increase PID activity in the presence of auxin (Benjamins et al., 2003). While phosphorylation of PBP1 has not been reported to be essential for protein function, PBP1 does contain two low stringency MAPK phosphorylation motifs. This suggests that PBP1 and PID, and hence polar auxin transport, could potentially be regulated, at least in part, by MAPK signaling modules involving MKK3. Another potential link between MKK3 and polar auxin transport should also be mentioned: *PIN7,* which encodes an auxin efflux protein involved polar auxin transport, was also down-regulated in the SALK 051970 T-DNA line. *PIN7* and *MKK3* show overlapping expression patterns and are expressed at similar levels (Genevestigator), consistent with some kind of functional relationship. Since PIN-family single loss-of-function mutants do not show abnormal phenotypes, analysis of *MKK3* expression patterns in *pin7* plants would likely not be fruitful. However PIN7 is closely related to PIN2, PIN3 and PIN4 (Paponov et al., 2005) and analysis of *MKK3* expression patterns in *pin3 pin7* and *pin2 pin 7* double mutants reported by Paponov et al (2005) could substantiate the relationship between MKK3 and PIN7.

Since *MKK3, PIN7, PID* and *PBP1* exhibit overlapping expression patterns, *MKK3, PID* and *PBP1* are all induced by auxin exposure, and *PBP1* expression appears to be linked with *MKK3* expression, it seems that MKK3 signaling is a likely component of a polar auxin transport-associated MAPK signaling module. Examination of PID kinase activity in SALK 051970 T-DNA insertion plants exposed to auxin could provide more insight into this putative role.

ARFs are transcription factors that can act either as transcriptional activators or repressors (Tiwari et al., 2003). Although they were first characterized as auxin-responsive genes, they are now known to be involved in several aspects of plant physiology including embryo development, and auxin-, ethylene- and jasmonate-associated signaling (Okushima et al., 2005). The function of these proteins is regulated by Aux/IAAs, proteins that interact with and sequester ARFs when auxin is absent or present in low concentrations (Okushima et al., 2005). In response to increasing concentrations of auxin, Aux/IAAs are targeted for ubiquitination and degradation, thus disrupting their interaction with their cognate ARFs (Tiwari et al., 2003), resulting in ARF-mediated changes in gene expression (Tiwari et al., 2003). *ARF9*, which was specifically down-regulated in the SALK 051970 T-DNA insertion line, encodes a protein that has been shown to function as a transcriptional repressor (Tiwari et al., 2003) in response to auxin, although it is not known which specific Aux/IAA(s) control ARF9 activity. Two T-DNA insertional mutants in the coding sequences of *ARF9* do not display abnormal growth phenotypes, although this is not surprising given that *ARF*s form a 23-member gene family (Okushima et al., 2005). Nonetheless, absence of ARF9 may confer an abnormal auxin response phenotype, and comparison of the auxin-induced transcript profiles of the SALK 051970 T-DNA insertion line and *arf9*-null mutants could give insight into the link between MKK3, ARF9 and auxin-induced signal transduction.

It is intriguing that *ACS6,* whose expression was down-regulated in the SALK 051970 T-DNA insertion line, was identified in the GO annotation as an auxin-responsive gene, since it is functionally involved in ethylene biosynthesis and has generally been characterized as stress-responsive (Arteca and Arteca, 1999; Liu and Zhang, 2004). However, a characteristic plant response to auxin exposure is increased ethylene production (Hansen and Grossmann, 2000).

Since ACS6 is involved in ethylene production, and is also a phosphorylation substrate of AtMPK6 (Liu and Zhang, 2004), it is possible that auxin-induced ethylene production in the SALK 051970 T-DNA insertion line may be impaired. Other links between MKK3 and ethylene signaling were observed in the microarray study, with four ERFs being down-regulated (Table 3.4).

Differentially regulated ABA-associated genes

The links between MKK3 function and ABA are particularly striking. The *MKK3* promoter sequences are responsive to ABA, and ABA-responsive CAREs are over-represented in the promoter regions of *MKK3* and many of the differentially regulated genes in the SALK 051970 T-DNA insertion line. When the list of mis-regulated genes in this line was examined for ABA-responsive genes, both multi-responsive and more specifically responding genes could be identified.

As previously discussed, ERF4 is involved in ABA, salt and ethylene signaling and the association of *MKK3* and *ERF4* expression could reflect a role of MKK3 in regulating cross-talk between stress-responsive hormone signaling modules. Another down-regulated gene, *ZAT10* (At1g27730), which was subsequently up-regulated upon over-expression of both full length *MKK3* and *MKK3ΔNTF2* variants in the SALK 051970 T-DNA insertion line, may provide another link between MKK3 signaling and multiple hormone pathways. *ZAT10* expression was initially reported to be responsive to salt, and ectopic expression of *ZAT10* in yeast resulted in increased salt tolerance (Lippuner et al., 1996). In the public microarray database, *ZAT10* expression is recorded as also being up-regulated by ethylene and ABA (Genevestigator), while more recently, *ZAT10* expression has been shown to be induced by the jasmonate precursor, OPDA, possibly in a wound-associated context (Taki et al., 2005).

Other ABA-associated genes identified in the microarray study appear to be specifically involved in ABA, salt and osmotic stimuli. The pattern of *AtHB7* expression in roots is similar to that of *MKK3*, with expression being detected primarily in root tips (Soderman et al., 2000); it is also induced by ABA (Soderman et al., 1996). Over-expression of *AtHB7* results in diminished elongation growth, while *AtHB7* anti-sense suppressed plants do not show a visible phenotype (Soderman et al., 2000). The coincident expression of *MKK3* and *AtHB7*, and the reported role of AtHB7 as a negative regulator of growth in response to water-stress and ABA exposure (Olsson et al., 2004), are both consistent with the proposed function of MKK3 as a regulator of growth in response to abiotic stress and phytohormones (Chapter 2), as is the observation that over-expression of *MKK3* and *MKK3ΔNTF2* in the SALK 051970 restored *AtHB7* expression to WT levels.

At3g11410 encodes AHG3, a protein phosphatase 2C (PP2C) that has recently been characterized as a negative regulator of ABA signaling (Yoshida et al., 2006). *ahg3-1*-null mutants are hypersensitive to ABA, and although untreated plants appear similar to WT, *ahg3-1* seedlings display increased growth inhibition in response to ABA (Yoshida et al., 2006). While I did not observe such a response in the SALK 051970 T-DNA insertion line, expression of *AHG3* is clearly associated with MKK3 since the down-regulated expression pattern of *AHG3* was reversed, and even slightly up-regulated, in both *MKK3*-variant over-expression lines.

Dehydrins such as ERD10 and COR47 are proteins that appear to be found in all photosynthetic organisms and are thought to have wide-ranging functions in stress-responses associated with ABA, salt and cold (Puhakainen et al., 2004). Some of the proposed functions of the various dehydrins include antifreeze activity, membrane stabilization, osmoregulation, free radical scavenging and acting as calcium-dependent chaperones (Puhakainen et al., 2004). The

expression of both *COR47* and *ERD10* is induced primarily by cold-stress, but also by ABA and salt (Puhakainen et al., 2004). Furthermore, pair-wise over-expression of either *COR47* and *RAB18* (another dehydrin) or *ERD10/LTI29* and *LTI30,* confers increased freezing tolerance (Puhakainen et al., 2004). However, no further functional characterization of these proteins has been reported. Nonetheless, diminished expression of multiple dehydrins in the SALK 015970 T-DNA insertion line suggests that it may be more susceptible to freezing conditions, which has yet to be examined. However, because the biochemical roles of these proteins in the ABA and salt response have not been characterized, it is possible that MKK3 signaling is associated with these functions and not with freezing-tolerance.

The model of MKK3 as a general organizer of the stress-response is supported by the down-regulation of *P5CS1* in the SALK 051970 T-DNA line. *P5CS1* encodes one of two P5CS (delta 1-pyrroline-5-carboxylate synthetase) enzymes in Arabidopsis, which catalyze an essential step in proline biosynthesis. While P5CS2 appears to be a housekeeping protein responsible for proline supply to the protein synthesis systems during embryonic and plant development, P5CS1 appears to be specifically involved in proline biosynthesis induced by environmental stresses (Szabados et al., 2005). *p5cs1* null mutants fail to accumulate proline as an osmolyte in response to osmotic stress and the mutant plants exhibit higher levels of ROS accumulation than do WT plants exposed to similar conditions (Szabados et al., 2005).

Differentially-regulated heat-responsive genes

Only two genes annotated as being associated with heat stress were differentially regulated in the SALK 051970 T-DNA insertion line. One of these, At4g21870 (HSP17) was up-regulated, and the other, At1g67970 (HSF5), was down-regulated. Although no functional data have been reported for HSP17, its expression pattern is the opposite of *MKK3* expression; *HSP17*

expression decreases >2-fold in response to salt, osmotic stress and auxin, and also appears to decrease slightly (1.5 fold) in response to heat-shock. Reciprocal conditional expression patterns, and up-regulation of *HSP17* expression in the *SALK* genotype, suggests that these gene products may be functionally related. Direct functional data are also not available for HSF5, but the expression patterns of *MKK3* and *HSF5* do not appear to overlap, the respective promoters do not appear to respond to similar treatments.

Function of the NTF2 domain

In an attempt to gain insight into the function of the NTF2 domain encoded by *MKK3,* I examined the expression profiles of 22 genes that were differentially regulated in the SALK 051970 T-DNA insertion line when that line was complemented by over-expression of either full-length *35S:MKK3* or *35S:MKK3ΔNTF2*. While these experiments illustrate the ability of each MKK3 variant to affect gene expression, I can not determine if these effects are due to ectopic expression of each variant or if they accurately represent the normal behaviour of MKK3-associated genes. Subsequent experiments examining the effect of re-expression of each MKK3 variant under the control of the endogenous MKK3 promoter sequences will clarify this issue. Nonetheless, sixteen of the 22 genes analysed were down-regulated in the SALK 051970 T-DNA insertion line, and following over-expression of either *MKK3* variant the expression pattern of only one of these genes was found to be entirely unaffected. Thirteen were either expressed at or above the levels observed in WT plants in both over-expression lines, while two were affected differently depending on the variant of *MKK3* being over-expressed. The expression of *ANAC29/NAP* (At1g69640) was affected by over-expression of both *MKK3* variants, but while over-expression of the *MKK3ΔNTF2* variant restored expression of *ANAC29/NAP* to WT levels, over-expression of full-length *MKK3* resulted in a 3.5 fold up-

regulation of *ANAC29/NAP*. This could suggest that the NTF2 domain is required for the proper control of *ANAC29/NAP* expression by MKK3, perhaps through the formation of a multi-protein complex mediated by the NTF2 domain. The NTF2 domain is known to form heterodimers in other organisms (Thakurta et al., 2004) but the interaction site between NTF2 homodimers, or between NTF2 domain-containing protein heterodimers, is defined by hydrophobic patches within the tertiary peptide structure. At this point, we lack the structural information needed to assess such a possible interaction between the MKK3 NTF2 domain and other proteins. In the absence of structural data, potential interactions between MKK3, MKK3ΔNTF2 and ANAC29/NAP-could be tested by *in vitro* or yeast two-hybrid methods.

Over-expression of the two *MKK3* variants had the opposite effect on the expression of At5g04340, which encodes a C2H2 zinc-finger transcription factor. This would suggest that the MKK3 NTF2 domain is required for proper expression of At5g04340. C2H2 zinc finger transcription factors, such as soybean SCOF-1, have been shown to be involved in the environmental stress-response (Kim et al., 2001). Over-expression of *SCOF-1* in Arabidopsis plants induced expression of COR (dehydrin) genes including *COR47,* a gene that was also down-regulated in the SALK 051970 T-DNA insertion line. Interestingly, the closest SCOF-1 homologues in Arabidopsis are ZAT10 and At5g04340. *ZAT10* expression was also affected both by elimination of MKK3 signaling and by subsequent over-expression of each *MKK3* variant, suggesting that MKK3, COR47, ZAT10 and At5g04340 are functionally related, likely in an environmental stress-associated role. Similar to *COR47* and *ZAT10,* At5g04340 expression is reported to be responsive to multiple environmental stresses, including salt, osmotic, cold, ABA and SA (Genevestigator), further substantiating the link between MKK3 and the expression of these genes. Furthermore, At5g04340 contains a putative MAPK substrate motif.

When six of the up-regulated genes in the SALK 051970 T-DNA line were also examined, three were found to be unresponsive to over-expression of either *MKK3* variant. Two of the remaining genes showed *MKK3*-variant-specific expression profiles and one was affected by both variants. Expression of At4g38840 (*SAUR*; Aux/IAA) and of At1g14210, which encodes a T2 ribonuclease, was only restored to WT expression levels in the *35S:MKK3ΔNTF2* line. The expression of *MKK3* and each of these genes appears to be inversely correlated in response to salt, ABA and osmotic stress (Chapter 2; Genevestigator). However, due to the very modest fold changes in the WT and full length over-expression lines, and poor statistical results for these genes in the *35S:MKK3ΔNTF2* lines, further analysis of their relationships, either through co-expression analysis in response to salt, osmotic and ABA treatments, or through expression profiling in At4g38840- or At1g14210-null mutants, needs to be conducted.

CONCLUSIONS

The characteristics of *MKK3* expression, including the response of the *MKK3* promoter to abiotic stresses and phytohormones, suggested that MKK3 signaling may be involved in floral development and in stress-induced phytohormone signaling modules (Chapter 2). The SALK 051970 T-DNA insertion line does not produce a full-length *MKK3* transcript, suggesting that this line might represent a *mkk3*-null mutant. While it appears unlikely, the persistence of a truncated *MKK3* transcript upstream of the T-DNA insertion, and the potential of that transcript to yield a MKK3 variant protein lacking the NTF2 domain, cannot be excluded. The differential ability of expression of full length *MKK3* and *MKK3ΔNTF2* variants to complement the transcriptional phenotype of the SALK 051970 line supports the notion that this line does represent an *mkk3*-null mutant. Attempts to identify a macroscopic phenotype that could be attributed to either the putative elimination of MKK3 signaling (in the SALK 051970 T-DNA

insertion line) or to ectopic expression of variant MKK3 proteins were unsuccessful. Nonetheless, expression profiling experiments using both the SALK 051970 T-DNA line and the *35S:MKK3* variant over-expression lines revealed several groups of genes that were mis-regulated in the SALK 051970 T-DNA insertion line. Many of these genes are associated with plant development, phytohormone signaling and stress responses. Furthermore, several of the mis-regulated genes are components of signaling pathways that are apparently involved in cross-talk between various phytohormone and stress responsive signaling pathways. This supports the suggested role of MKK3 as a general organizer of the stress-response at the phytohormone signaling level. While it appears that the NTF2 domain is important for the proper control of expression of some genes, a clear pattern amongst NTF2 domain-dependent genes could not be discerned and similar classes of genes fall into the NTF2-dependent and NTF2-independent categories. Diminished steady-state levels in the SALK 051970 T-DNA insertion plants of a significant number of genes associated with stress responses would suggest that these plants may not be well equipped to cope with environmental stresses. However, I was unable to find any experimental evidence for this in relatively short-term experiments. Nonetheless, since the transcriptional phenotype of the SALK 051970 T-DNA insertion line was determined using untreated plant samples, it would be informative to characterize the transcriptome profiles of the SALK 051970 T-DNA insertion line after challenge with several stimuli including auxin, ABA, salt and cold, and to monitor the fitness of these plants over the longer term in stressful environments, such as field-level survival studies.

CHAPTER 4. Analysis of MKK3 protein function

INTRODUCTION

Signaling through a mitogen-activated protein kinase signaling module is activated by the sequential phosphorylation and activation of a MAPK kinase kinase (MAPKKK), MAPK kinase (MAPKK) and finally a MAPK. MAPKKs thus play central roles in these cascades, since they connect the numerous upstream MAPKKKs to the cascade output components, the MAPKs. In Arabidopsis, MKK3 is a particularly interesting MAPKK since, in addition to the archetypical dual-specificity kinase domain, it also contains an NTF2 domain. At the time that this work was initiated, the biological roles of MKK3, and the function of its NTF2 domain, were unknown. Identification of all interacting proteins and substrates of any given MAPKK would provide important insights into the organization of the modules in which it participates, and the extent of cross-talk that occurs between signal pathways. At the same time, manipulation of the protein domain structure can allow at least an initial assessment of that domain's contribution to the function of the protein. In view of the large number of proteins produced in any eukaryotic cell, and the relatively simple motif (-S/TP-) that appears to define the phosphorylation site of MAPK substrates, identification of the targets of MAPK signal modules can be challenging. One approach to defining these connections is to activate signaling through a module and characterize the biological outcomes of that activation. However, for virtually all MAPK modules in plants, the upstream activating MAPKKKs have yet to be defined, which limits the opportunity to initiate module signaling at that level. In the absence of direct knowledge of an upstream activation stimulus, signaling though the lower components of a MAPK signaling module can be artificially activated at the MAPKK level by introduction of a "constitutively active" version of

the appropriate MAPKK. Substitution of the serine or threonine residues in the S/TXXXXXS/T activation loop of a MAPKK with the acidic amino acids, glutamate or aspartate, mimics phosphorylation of those sites and thereby creates a permanently active form of the enzyme (Mansour et al., 1994; Yang et al., 2001).

This approach has been used to study specific plant MAPK signaling modules both *in vitro* and *in vivo* (Zhang and Liu, 2001; Cluis, 2005). However, when CA-MAPKKs are to be examined *in vivo*, some method of controlling the transgene's expression is important, particularly if the goal is generation of viable transgenic lines. Since a CA-MAPKK represents a dominant trait when the corresponding transgene is inserted into a plant, and constitutive activation of MAPK modules has the potential to be lethal, expression of CA-MAPKK constructs is usually placed under the control of an inducible promoter system (Liu et al., 2003; Cluis, 2005). Induction of CA-MAPKK expression at the desired time then allows the full range of phenotypic consequences of this signaling, including gene expression changes, to be examined.

Other methods of identifying specific components of MAPK signaling modules include yeast two-hybrid analysis or co-immunoprecipitation to identify interacting proteins, and *in vitro* phosphorylation assays to identify direct substrates of the kinases (Ichimura et al., 1998; Zhang and Liu, 2001; Soyano et al., 2003; Feilner et al., 2005). As part of my characterization of AtMKK3, I assessed the ability of MKK3 and of a MKK3ΔNTF2 variant to interact with Arabidopsis MAPKs via yeast two-hybrid analysis, and examined the ability of 'constitutively active' versions of these MKK3 variants to activate recombinant, purified MAPKs, using *in vitro* activation assays. Furthermore, I used transgenic Arabidopsis plants carrying a dexamethasone-inducible, constitutively active *MKK3* (*CA-MKK3*) construct to analyze the phenotypic

consequence of activating MKK3 signaling modules at various points throughout plant development.

MATERIALS AND METHODS

Cloning of *MKK3* and *MKK3ΔNTF2* into the Gateway™ Entry Vector *pCR8*

In order to facilitate further modifications of the *MKK3* coding sequences the previously described full-length *MKK3* and *MKK3ΔNTF2* variants (Chapter 3, Materials and Methods) were cloned into the Gateway™ Entry Vector *pCR8*. Because both full-length *MKK3* and *MKK3ΔNTF2* clones contain the same initiation and termination sequences, both clones were amplified from the respective parent vector using the PCR primers MKK3FL-F and MKK3-3HA-R primers and Platinum Taq HIFI (Invitrogen, Burlington, ON, Canada) and cloned by TOPO-TA mediated cloning into *pCR8*, generating the vector *pMKK3-ENTRY* and *pMKK3ΔNTF2-ENTRY*. The accuracy of both sequences was verified by DNA sequence analysis prior to further manipulation (NAPS, UBC).

Generation of *pMKK3-DEST32* and *pMKK3ΔNTF2-DEST32* bait vectors for yeast two-hybrid analysis

pMKK3-ENTRY and *pMKK3ΔNTF2-ENTRY* were each used as a donor vector in Gateway™ LR-clonase mediated recombination reactions to shuttle the full-length *MKK3* and *MKK3ΔNTF2* variants into the yeast two-hybrid bait vector *pDEST32* (Invitrogen, Burlington, ON, Canada; Figure 4.1). Briefly, a 20 µL recombination reaction (150 ng donor vector (*pMKK3-ENTRY* or *pMKK3ΔNTF2-ENTRY*); 300 ng destination vector (*pDEST32*); 4 µL LR Clonase (Invitrogen, Burlington, ON, Canada)) in 1X TE buffer was set-up and incubated at 25°C for one hour.

Afterwards, residual Clonase enzyme was digested by adding 2 μL proteinase K solution (Invitrogen, Burlington, ON, Canada) and incubating the samples at 37°C for ten minutes. Recombinant plasmids resulting from these reactions were introduced into *E. coli* DH5α using 5 μL of each recombination reaction, and transformed cells were plated on LB agar plates containing gentamicin (10 μg/mL) for incubation overnight at 37°C. Plasmid DNA from positive colonies growing in the presence of gentamicin was isolated using the QiaPrep DNA Miniprep kit (Qiagen, Mississauga, ON, Canada) and sequenced to ensure maintenance of the *MKK3*-variant DNA sequences upon recombination into the recipient vector, *pDEST32*.

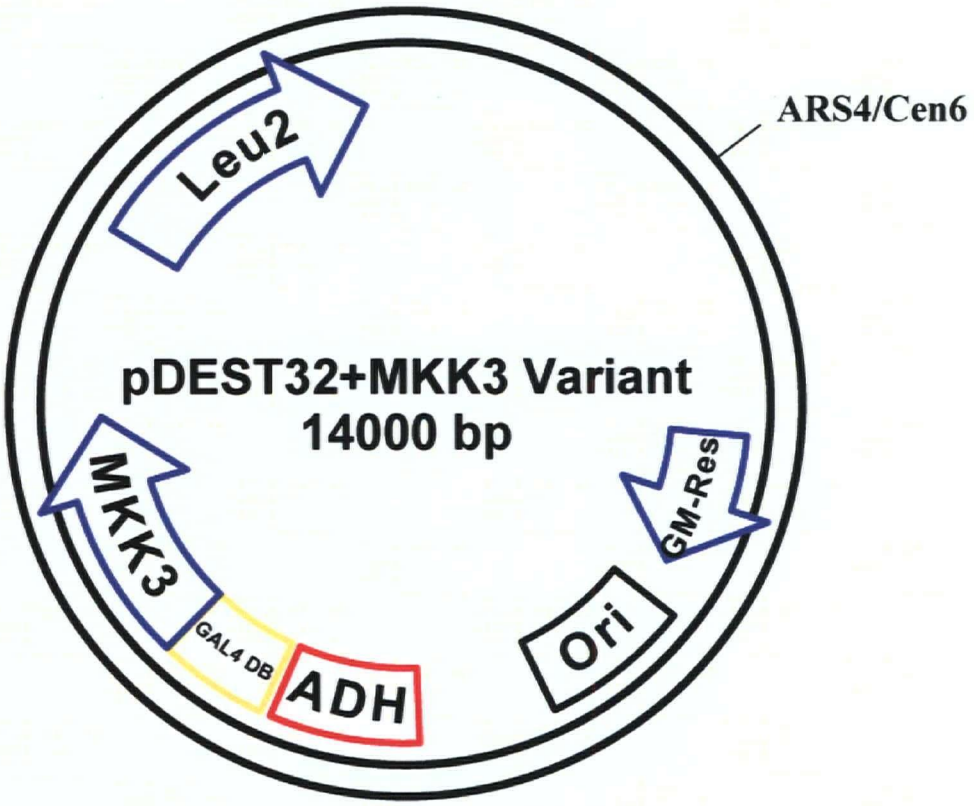

Figure 4.1. Schematic representation of *pDEST32* containing an *MKK3* variant insert. These vectors served as bait vectors for yeast two-hybrid analysis of MKK3 variant – AtMPK interactions. The *MKK3* variants were inserted as an N-terminal translational fusion to the GAL4 DNA binding domain, expression of which was controlled by the ADH promoter. Transformed yeast strains were selected for on the basis of growth in minimal media lacking leucine. GM = gentamicin resistance gene.

Generation of MAPK prey vectors for yeast two-hybrid analysis

The Arabidopsis genome encodes twenty distinct MAPKs (Ichimura et al., 2002; Hamel et al., 2006). Sequences encoding each MAPK, with the exception of *MPK15* and *MPK19,* were isolated from Arabidopsis cDNA in the Ellis Research Group and cloned into the Gateway™ entry vector *pCR8* via TOPO-TA mediated cloning as previously described. Each cloned MAPK

was sequence-verified to ensure integrity of the cloned gene prior to their transfer into the Gateway™-compatible yeast two-hybrid prey vector *pDEST22*. These sequence-verified entry vector clones form part of the Ellis Research Group clone set. Recombination reactions were carried out as described above (Generation of *pMKK3-DEST32* and *pMKK3ΔNTF2-DEST32* bait vectors for yeast two-hybrid analysis) and again, the MAPK sequence in each prey vector was verified by DNA sequence analysis prior to transformation of yeast strains (NAPS, UBC; Figure 4.2).

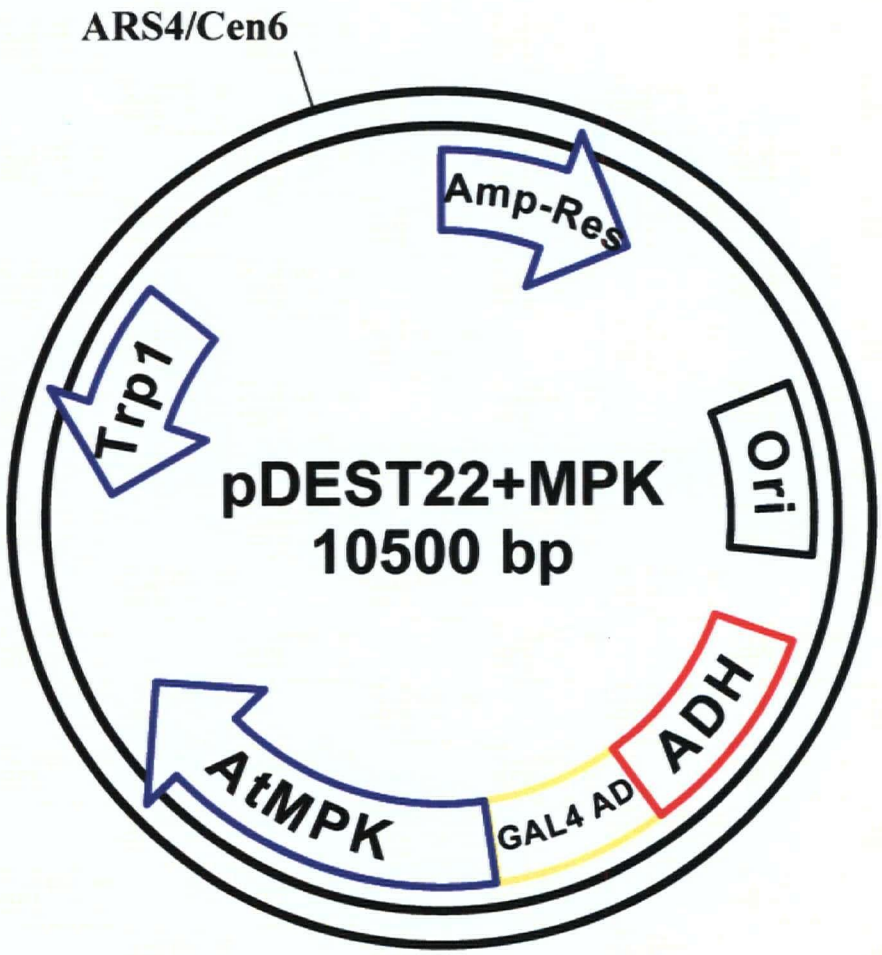

Figure 4.2. Schematic representation of *pDEST22* prey vector containing an Arabidopsis MAPK (AtMPK). These vectors served as prey vectors for yeast two-hybrid analysis of MKK3 variant – AtMPK interactions. Individual *MPK*s were inserted as N-terminal translational fusions to the GAL4 activation domain which was controlled by the ADH promoter. Transformed yeast strains were selected for on the basis of growth in minimal media lacking tryptophan. Amp-res = ampicillin resistance gene.

Yeast two-hybrid analysis of MKK3 - MAPK interactions

Yeast two-hybrid analyses were used to determine the ability of MKK3 and MKK3ΔNTF2 to interact with each of the Arabidopsis MAPKs contained within the Ellis Research Group clone

set using the ProQuest Yeast Two-hybrid System (Invitrogen, Burlington, ON, Canada). The yeast strain MaV203 (MAT α; MaV203 (MAT α, leu2-3,112, trp1-901, his3Δ200, ade2-101, gal4Δ, gal80Δ, SPAL10::URA3, GAL1::lacZ, HIS3UAS GAL1::HIS3@LYS2, can1R, cyh2R) was serially transformed, first with the appropriate *pDEST-22-MAPK* prey vector (containing the GAL4 activation domain), followed by the appropriate *pDEST-32-MKK3* variant bait vector (containing the GAL4 DNA binding domain). Transformants capable of growing in the absence of both leucine and tryptophan, indicating the presence of both bait and prey vectors, were subsequently screened for interactions between the MKK3 variant and MAPK using three different selection criteria: growth in medium lacking histidine (SC-leu-trp-his; Appendix 2), growth in the absence of uracil (SC-leu-trp-ura; Appendix 2) and failure to grow in the presence of 5-fluoroorotic acid (5-FOA; SC-leu-trp+FOA; Appendix 2). Briefly, overnight cultures of the MaV203 strain co-transformed with either MKK3 or MKK3ΔNTF2 and a specific MAPK (e.g. MKK3 + MPK1) grown in SC-leu-trp media were diluted 1:1 000 in PBS. Diluted cultures were spotted on duplicate agar plates containing each of four media types (SC-leu-trp, SC-leu-trp-his+3AT, SC-leu-trp-ura, SC-leu-trp+5FOA) and growth at 30°C was monitored over a 48-hour period. Growth in the respective media types was scored and these data were interpreted to determine the presence of protein-protein interactions.

Generation of a 'constitutively active' variant of MKK3

To generate sequences encoding a 'constitutively active' variant of MKK3 (CA-MKK3), the protein sequences of MKK2, MKK3, MKK4, MKK5 and NtMEK2 were aligned in order to identify the -S/TXXXXXS/T- activation loop (Figure 4.3; Ichimura et al., 1998). Site-directed mutagenesis was carried out using the QuikChange II site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA). Briefly, two complementary PCR primers, CAKK3SDMF (GGC ATA

AGT GCT GGC CTT GAG AAT GAA ATG GCT ATG TGT GCT GAT TTT GTT GGA ACT GTC ACC TAC ATG TCA CC) and CAKK3SDM-R (GGT GAC ATG TAG GTG ACA GTT CCA ACA AAA TCA GCA CAC ATA GCC ATT TCA TTC TCA AGG CCA GCA CTT ATG CC), containing the desired mutation were synthesized (NAPS, UBC). These primers possessed a melting temperature >80°C, which is required for efficient mutagenesis, and they contained sequences that would change the –**S**MAMCA**T-** sequence encoded by the endogenous *MKK3* gene to –**E**MAMCA**D-**. A 50 µL mutagenesis reaction (5X reaction buffer (Stratagene, La Jolla, CA, USA); 25 ng plasmid DNA template; 125 ng each of forward and reverse mutagenic primer; 1 µL dNTP mixture (Stratagene, La Jolla, CA, USA); 5 units *pfu* Ultra DNA polymerase (Stratagene, La Jolla, CA, USA)) was set-up and mutagenized plasmid was synthesized in a thermocycler using 16 cycling reactions (95°C X 30 sec; 55°C X 60 sec; 68°C X 1 min/kb template plasmid length; 5 minutes for full-length MKK3 and 4.5 minutes for the MKK3ΔNTF2 variant). Parental template DNA was digested with *Dpn*I at 37°C for one hour prior to transformation of competent *E. coli* XL-10® Blue Supercompetent cells (Stratagene, La Jolla, CA, USA). Transformation reactions were plated on LB agar plates containing ampicillin (100 µg/mL) and incubated at 37°C overnight. Ampicillin-resistant colonies were cultured overnight in LB broth containing ampicillin (100 µg/mL) and plasmid DNA was extracted using the QiaPrep DNA Miniprep kit (Qiagen, Mississauga, ON, Canada). Successful mutagenesis was verified by DNA sequence analysis, with the complete *MKK3*-variant sequence being verified to ensure no additional modifications had been made to the coding sequences.

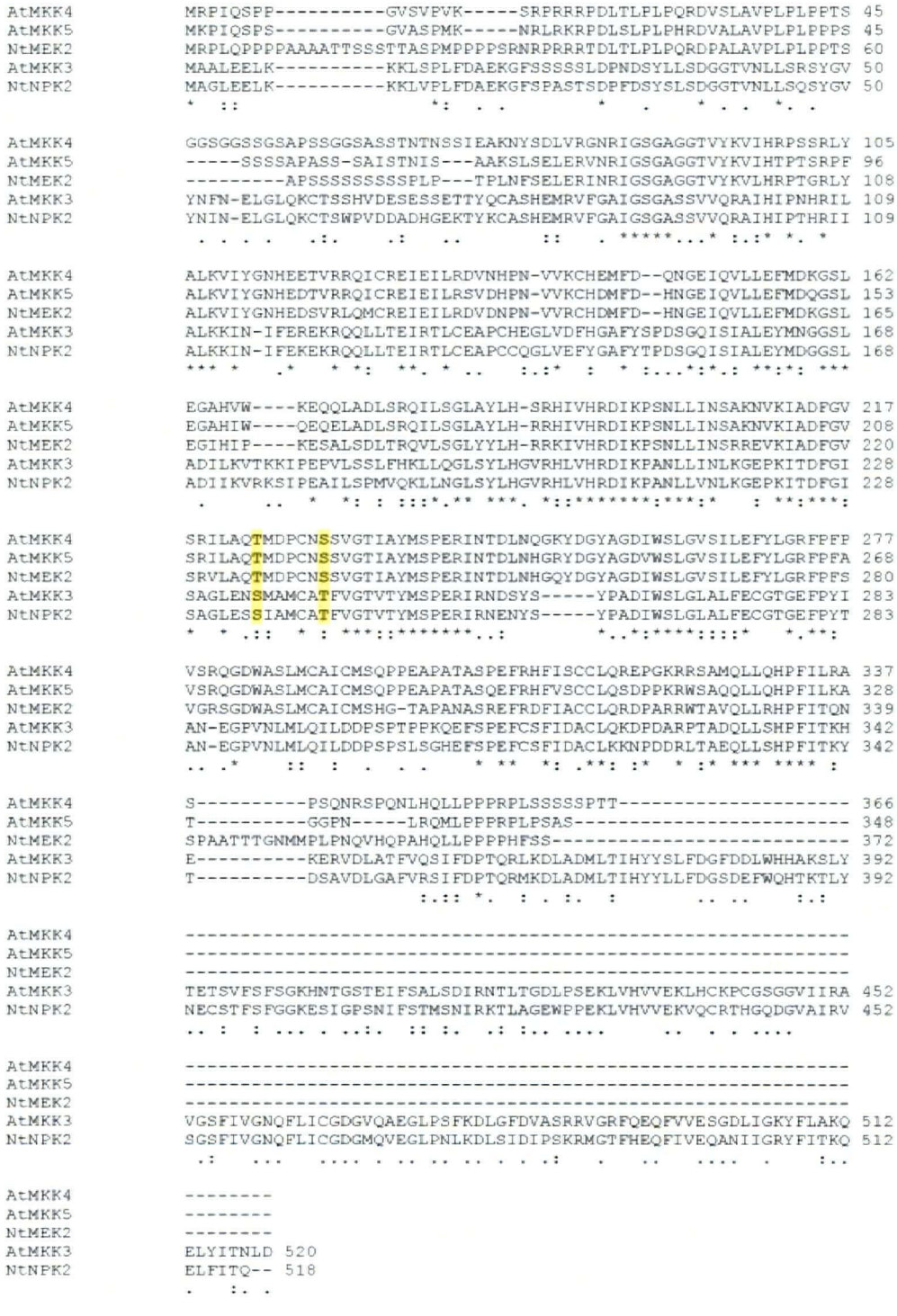

Figure 4.3. ClustalW (1.82) multiple sequence alignment of MAPKKs. MAPKK protein sequences of AtMKK3, 4 and 5 along with NtMEK2 and NtNPK2 were aligned to identify the location of the -S/TXXXXXS/T- motif in MKK3 (yellow highlight).

Generation of poly-His-tagged variants of CA-MKK3 for recombinant protein production

To generate recombinant, 'constitutively active' MKK3 (CA-MKK3) and MKK3ΔNTF2 protein for *in vitro* activation studies, the sequences encoding *MKK3* and *MKK3ΔNTF2* contained in the Gateway™ entry vectors *pMKK3-ENTRY* and *pMKK3ΔNTF2-ENTRY* were transferred into the Gateway™-compatible *E. coli* IPTG-inducible expression vector *pEXP1-DEST* using Gateway™ recombination reactions as previously described (Generation of *pMKK3-DEST32* and *pMKK3ΔNTF2-DEST32* bait vectors for yeast two-hybrid analysis). Sequence integrity of both *MKK3* and *MKK3ΔNTF2* in *pEXP1-DEST* was verified by DNA sequence analysis prior to recombinant protein production.

Production and purification of recombinant proteins for *in vitro* substrate analysis

Recombinant protein production in E. coli *BL21 cells*

A series of *in vitro* phosphorylation studies were employed to try to identify MAPK substrates of *MKK3* and *MKK3ΔNTF2*, using affinity-purified recombinant MKK3 variants and MAPK proteins. Sequences encoding each MAPK (with the exception of *MPK15* and *MPK19*) had been previously cloned as an N-terminal glutathione-S-transferase (GST) fusion in the *E. coli* expression vector *pGEX-4T1* (Amersham, Baie d'Urfé, PQ, Canada), while sequences encoding each MKK3 variant were contained within the previously described *pEXP1-DEST* vector. These plasmids were introduced into the *E. coli* BL21 strain for recombinant protein expression and subsequent purification.

Recombinant proteins were expressed as follows: A 3 mL overnight culture in YTA media was diluted 1:100 in 250 mL YTA broth pre-heated to 37°C. The resultant cell suspension was allowed to grow, while shaking at 250 rpm at 37°C until an OD_{600} of 0.5-1.0 was reached. At this point, gene expression was induced by the addition of IPTG (0.5 mM final concentration) followed by continued growth while shaking at 250 rpm at 25°C for four hours. *E. coli* cells were harvested by centrifugation at 4 000 x g for 10 minutes and stored at -80°C until recombinant protein purification.

Purification of GST-tagged MAPK protein

GST-tagged MAPK proteins were purified batch-style using glutathione-Sepharose 4B beads (Amersham, Baie d'Urfé, PQ, Canada) as follows: Cell pellets of IPTG-induced *E. coli* BL21 cells were thawed on ice and resuspended in 25 mL ice-cold PBS. The resultant cell suspension was lysed by five successive 20-second sonication cycles, with the suspension being returned to ice for thirty seconds following each cycle. Triton X-100 (1% v/v) was added to the lysed cell mixture and the suspension was rocked gently at 4°C for 30 minutes. Cell debris was pelleted by centrifugation at 12 000 X g for 10 minutes at 4 °C, and the supernatant was transferred to 50 mL polypropylene tubes, followed by the addition of 500 µL equilibrated glutathione-Sepharose 4B slurry (50% v/v glutathione-Sepharose 4B beads in ice-cold PBS). GST-tagged proteins were allowed to bind to the beads at room temperature for two hours, after which protein-loaded beads were pelleted by centrifugation (500 x g) for 5 minutes at room temperature. The beads were washed three times with 10 mL PBS and pelleted each time. Elution of recombinant protein was carried out using two successive ten minute incubations in one mL elution buffer (10 mM reduced glutathione). Each eluate was pooled and stored at -80°C until further use.

Purification of His_6-tagged recombinant CA-MKK3 and CA-MKK3ΔNTF2 protein

Recombinant CA-MKK3 and CA-MKK3ΔNTF2 proteins were purified batch-style using Ni-NTA resin (Qiagen, Mississauga, ON, Canada), following the same protocol as that used for purification of GST-tagged proteins, with these exceptions: Cell pellets were resuspended in 20 mL native binding buffer (NBB; Appendix 2), all washes were conducted using native wash buffer (NWB; Appendix 2) and proteins were eluted in 1 mL native elution buffer containing imidazole (NEB; Appendix 2). Recombinant proteins were buffer exchanged with NBB and stored at -80°C until further use.

Identification of MKK3 and MKK3ΔNTF2 substrates by *in vitro* activation assays

The MAPK substrates of MKK3 and MKK3ΔNTF2 were examined using a series of indirect *in vitro* activation assays. MAPK activation resulting from phosphorylation in the presence of CA-MKK3 or CA-MKK3ΔNTF2 was determined by the analyzing the ability of activated MAPKs to phosphorylate the general MAPK substrate, myelin basic protein (MBP; Sigma-Aldrich, Oakville, ON, Canada). The extent of MBP phosphorylation was assayed via western blot analysis, using a monoclonal anti-phospho-MBP antibody (Upstate Cell Signaling, Charlottesville, VA, USA) that specifically recognizes the phosphorylated form of MBP. Each affinity-purified recombinant MAPK was incubated in the presence of ATP, either alone (to detect possible self-activation) or in the presence of either CA-MKK3 or CA-MKK3ΔNTF2. Phosphorylation reactions (2X kinase buffer (25 mM Tris, pH 7.5; 5 mM Beta-glycerophosphate; 2 mM DTT; 0.1 mM sodium orthovanadate; 10 mM $MgCl_2$; 200 μM ATP; MBP 5 mg/mL); 1 μg MAPK; 500 ng MAPKK) were carried out at 30 °C for one hour and were

stopped by the addition of 6X SDS-PAGE sample buffer (Appendix 2), followed by incubation at 100°C for ten minutes. Samples were separated on 12% PAGE gels, transferred to PVDF membranes and blocked in 5% skim milk in 1X TBST buffer (Appendix 2) for two hours at room temperature. Membrane-bound proteins were probed with anti-phospho-MBP as the primary antibody at a 1:1500 dilution in 2% skim milk in 1X TBST, overnight at 4°C. Following four successive five minute washes with 1X TBST, blots were probed with the secondary antibody, horseradish peroxidase-coupled anti-mouse IgG at a 1:8000 dilution in 1% skim milk in 1X TBST for two hours at room temperature. Blots were then washed three times for five minutes each in 1X TBST prior to reacting with the chemiluminescent detection reagent, ECL (Amersham, Baie d'Urfé, PQ, Canada), for 30 seconds in the dark followed by exposure to film.

Creation of transgenic Arabidopsis plants expressing *CA-MKK3* under the control of a dexamethasone-inducible promoter

The *CA-MKK3* variant carrying a triple HA tag was amplified by PCR using Platinum Taq HIFI and MKK3-F and MKK3-3HA-R primers, which include a 5' *Xho*I restriction site and 3' *Spe*I restriction site, respectively. The resultant PCR product was digested with *Xho*I and *Spe*I, gel-purified using the QiaQuik gel extraction kit (Qiagen, Mississauga, ON, Canada) and ligated to a previously *XhoI/SpeI* double-digested, dephosphorylated *pTA7002* vector, thus creating *pDex-CAKK3* (Figure 4.4). Ligation products were introduced into competent *E. coli* DH5α cells and the resultant kanamycin-resistant colonies were screened for the presence of correctly ligated *pDex-CAKK3* plasmid DNA. Insertion of sequences encoding CA-MKK3 was initially screened for by PCR analysis, using MKK3-F and pTA7002-R primers, and selected positive clones were confirmed by DNA sequence analysis (NAPS, UBC).

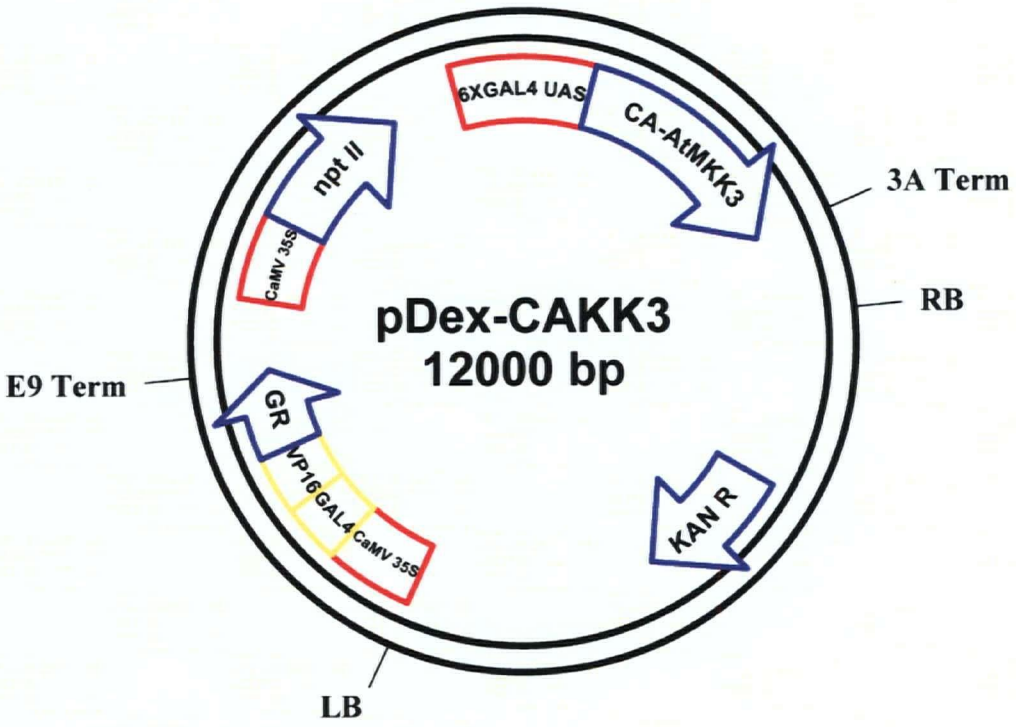

Figure 4.4. *pDex-CAKK3* binary vector used to create dexamethasone-inducible CA-MKK3 transgenic Arabidopsis plants. *CA-MKK3* sequences were inserted downstream of the 6XFAL4 UAS promoter that becomes active upon binding of the dexamethasone-bound chimeric GAL4-VP16-GR transcription factor. nptII = hygromycin resistance gene; KAN R = kanamycin resistance gene.

The *pDex-CAKK3* construct was introduced into Arabidopsis plants via the floral dip method using *Agrobacterium tumefaciens* strain EHA105. Transformed plants were identified on the basis of hygromycin B resistance and T2 plants were analysed for induction of *CA-MKK3* expression following treatment with dexamethasone as described previously (Chapter 3). Transgenic lines showing the highest levels of *CA-MKK3* expression were carried through to the T3 generation for the isolation of homozygous lines.

RESULTS

Yeast two-hybrid analysis of MKK3 – MAPK interactions

The goals of the MKK3 variant-MAPK protein interaction study were two-fold. First, I wanted to identify components of MAPK signaling modules in which MKK3 might function, and second, I hoped to gain further insight into the function of the NTF2 domain encoded by *MKK3*. On the basis of all three reporter systems (Figure 4.5 – 4.10), the yeast two-hybrid screen showed that full-length MKK3 is capable of interacting with MPK1, MPK2 and MPK7. The growth pattern of the relevant yeast colonies suggests that the interaction between MKK3 and MPK7 is stronger than the MKK3-MPK1 and MKK3-MPK2 interactions (Figure 4.5). By contrast, the MKK3ΔNTF2 variant was not found to interact with any of the MAPKs included in this study, including MPK1, MPK2 and MPK7 (Figure 4.8; Figure 4.9; Figure 4.10).

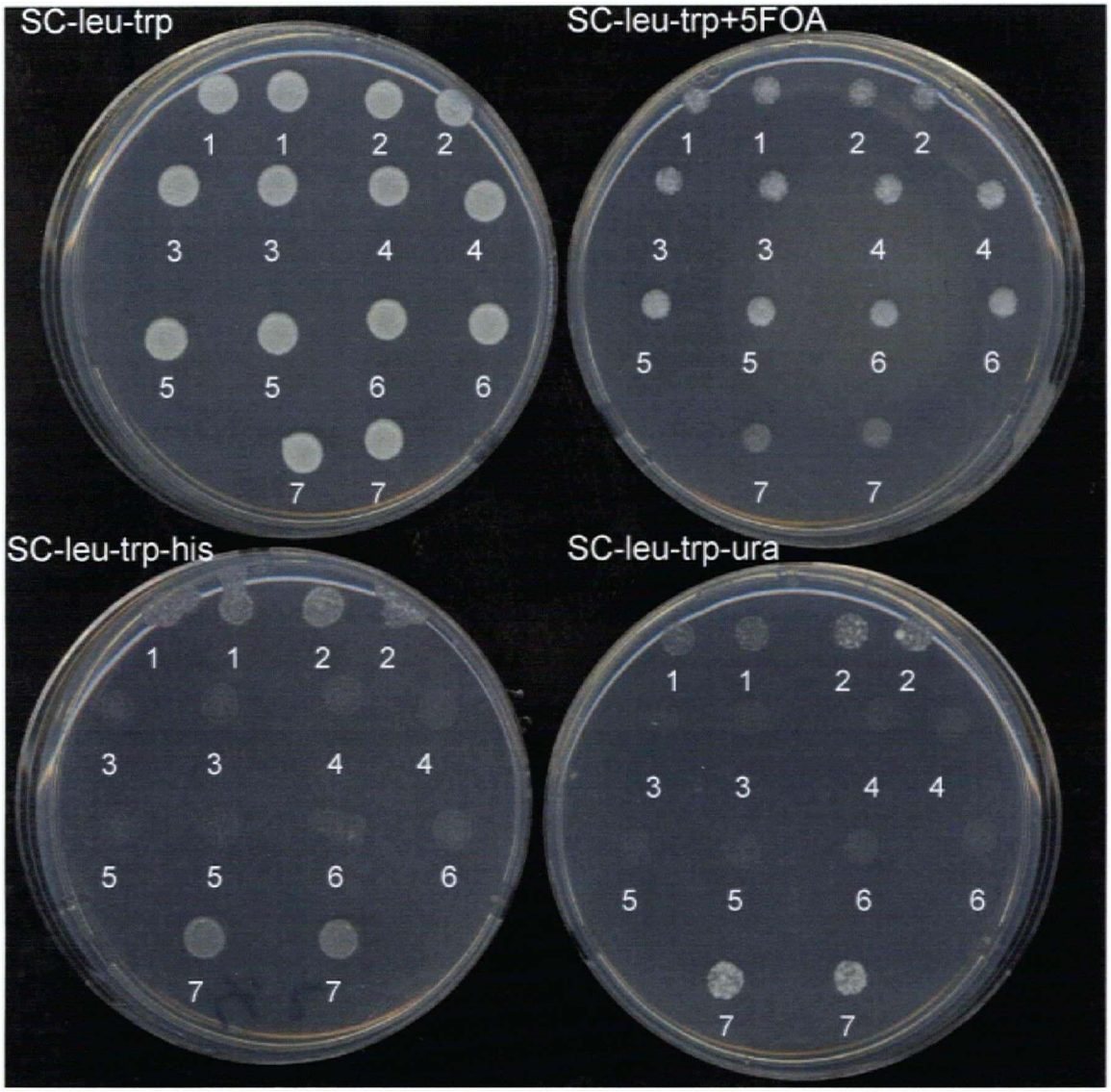

Figure 4.5. Yeast two-hybrid screening of full-length MKK3 + MPK1-7. Overnight cultures of the MaV203 strain co-transformed with MKK3 and a specific MAPK (MPK1-7) grown in SC-leu-trp media were diluted 1:1 000 in PBS. Diluted cultures were spotted on duplicate agar plates containing each of four media types (SC-leu-trp, SC-leu-trp-his+3AT, SC-leu-trp-ura, SC-leu-trp+5FOA) and growth at 30°C was monitored over a 48 hour period. Growth of yeast strains containing the combination of MKK3 + MPK1 (1), MKK3 + MPK2 (2) and MKK3 + MPK7 (7) on SC-leu-trp-his+3AT and SC-leu-trp-ura media types indicates the ability of each protein combination to interact. Growth inhibition of these combinations on SC-leu-trp+5FOA media verifies this interaction.

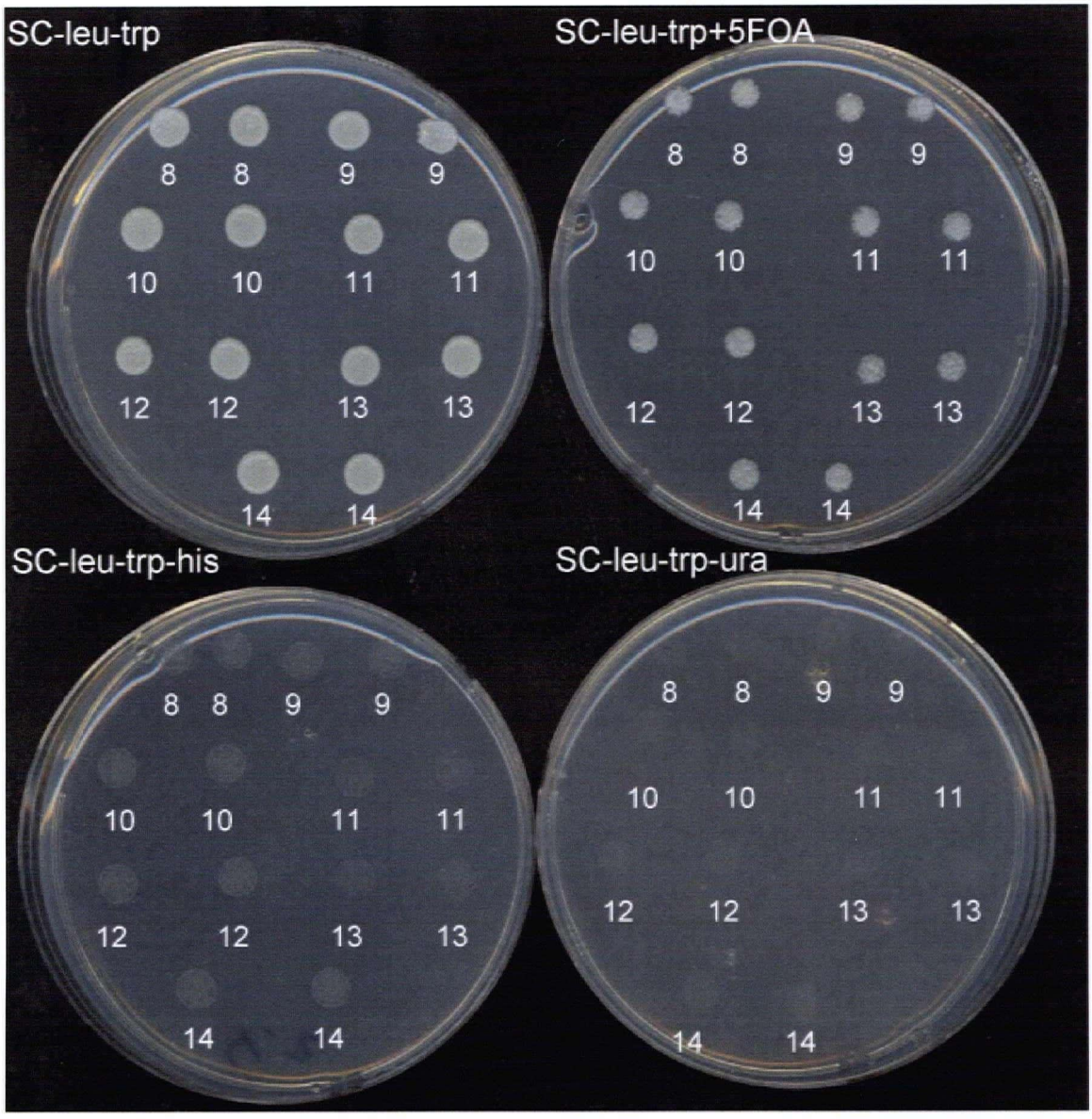

Figure 4.6. Yeast two-hybrid screening of full-length MKK3 + MPK8-14. Overnight cultures of the MaV203 strain co-transformed with MKK3 and a specific MAPK (MPK8-14) grown in SC-leu-trp media were diluted 1:1 000 in PBS. Diluted cultures were spotted on duplicate agar plates containing each of four media types (SC-leu-trp, SC-leu-trp-his+3AT, SC-leu-trp-ura, SC-leu-trp+5FOA) and growth at 30°C was monitored over a 48 hour period. Growth of all combinations on SC-leu-trp media verifies that the yeast strains contain both bait and prey plasmids. The lack of growth of any combination on SC-leu-trp-his+3AT and SC-leu-trp-ura media indicates the inability of those protein combination to interact. Lack of growth inhibition of all combinations on SC-leu-trp+5FOA media verifies these results.

Figure 4.7. Yeast two-hybrid screening of full-length MKK3 + MPK16, 17, 18 and 20. Overnight cultures of the MaV203 strain co-transformed with MKK3 and a specific MAPK (MPK16, 17 18 and 20) grown in SC-leu-trp media were diluted 1:1 000 in PBS. Diluted cultures were spotted on duplicate agar plates containing each of four media types (SC-leu-trp, SC-leu-trp-his+3AT, SC-leu-trp-ura, SC-leu-trp+5FOA) and growth at 30°C was monitored over a 48 hour period. Growth of all combinations on SC-leu-trp media verifies yeast strains contain both bait and prey plasmids. The lack of growth of any combination on SC-leu-trp-his+3AT and SC-leu-trp-ura media types indicates the inability of these proteins combination to interact. Lack of growth inhibition of all combinations on SC-leu-trp+5FOA media verifies these results.

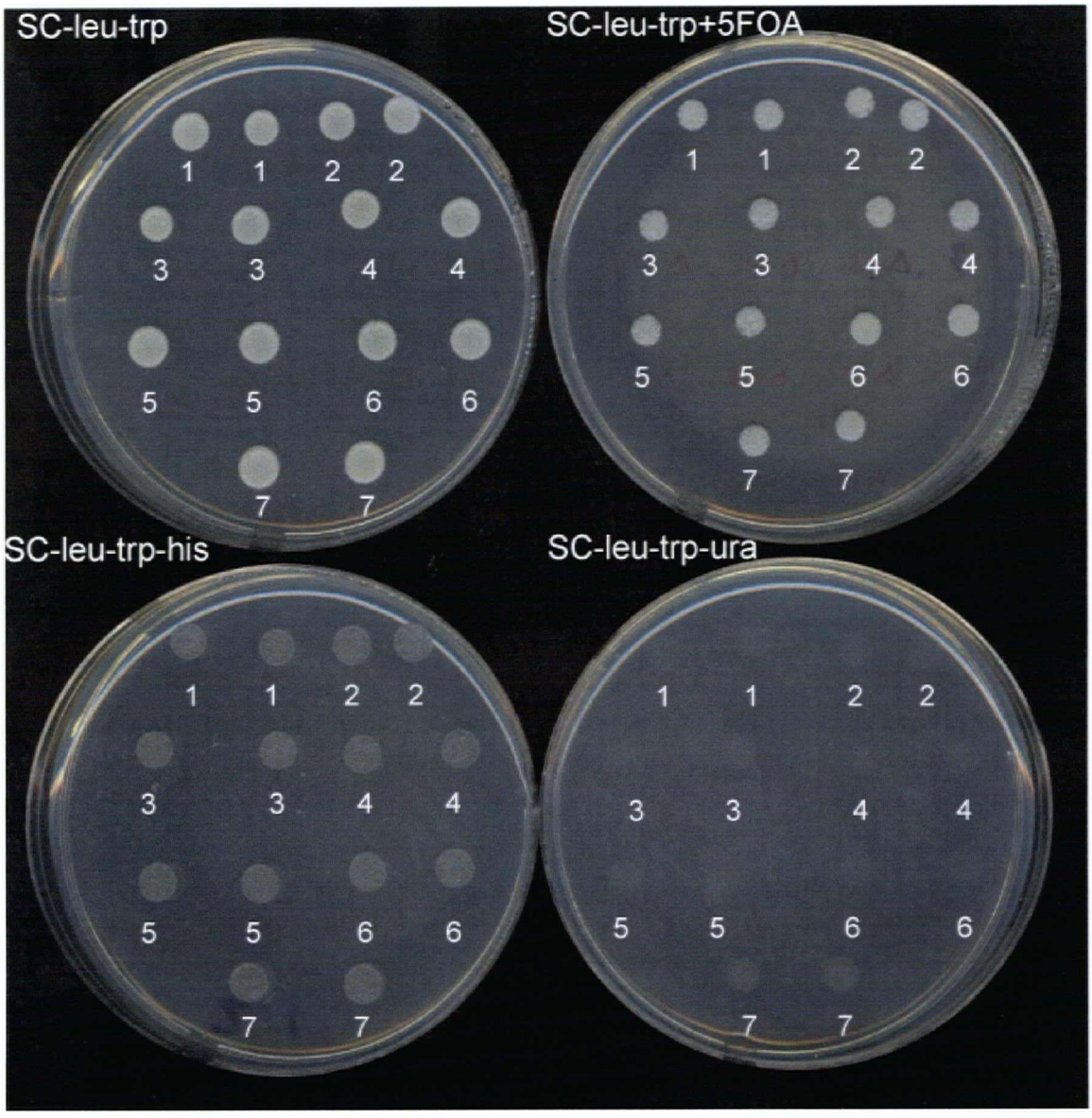

Figure 4.8. Yeast two-hybrid screening of MKK3ΔNTF2 + MPK1-7. Overnight cultures of the MaV203 strain co-transformed with MKK3ΔNTF2 and a specific MAPK (MPK1-7) grown in SC-leu-trp media were diluted 1:1 000 in PBS. Diluted cultures were spotted on duplicate agar plates containing each of four media types (SC-leu-trp, SC-leu-trp-his+3AT, SC-leu-trp-ura, SC-leu-trp+5FOA) and growth at 30°C was monitored over a 48 hour period. Growth of all combinations on SC-leu-trp media verifies yeast strains contain both bait and prey plasmids. The lack of growth of any combination on SC-leu-trp-his+3AT and SC-leu-trp-ura media types indicates the inability of each protein combination to interact. Lack of growth inhibition of all combinations on SC-leu-trp+5FOA media verifies these results.

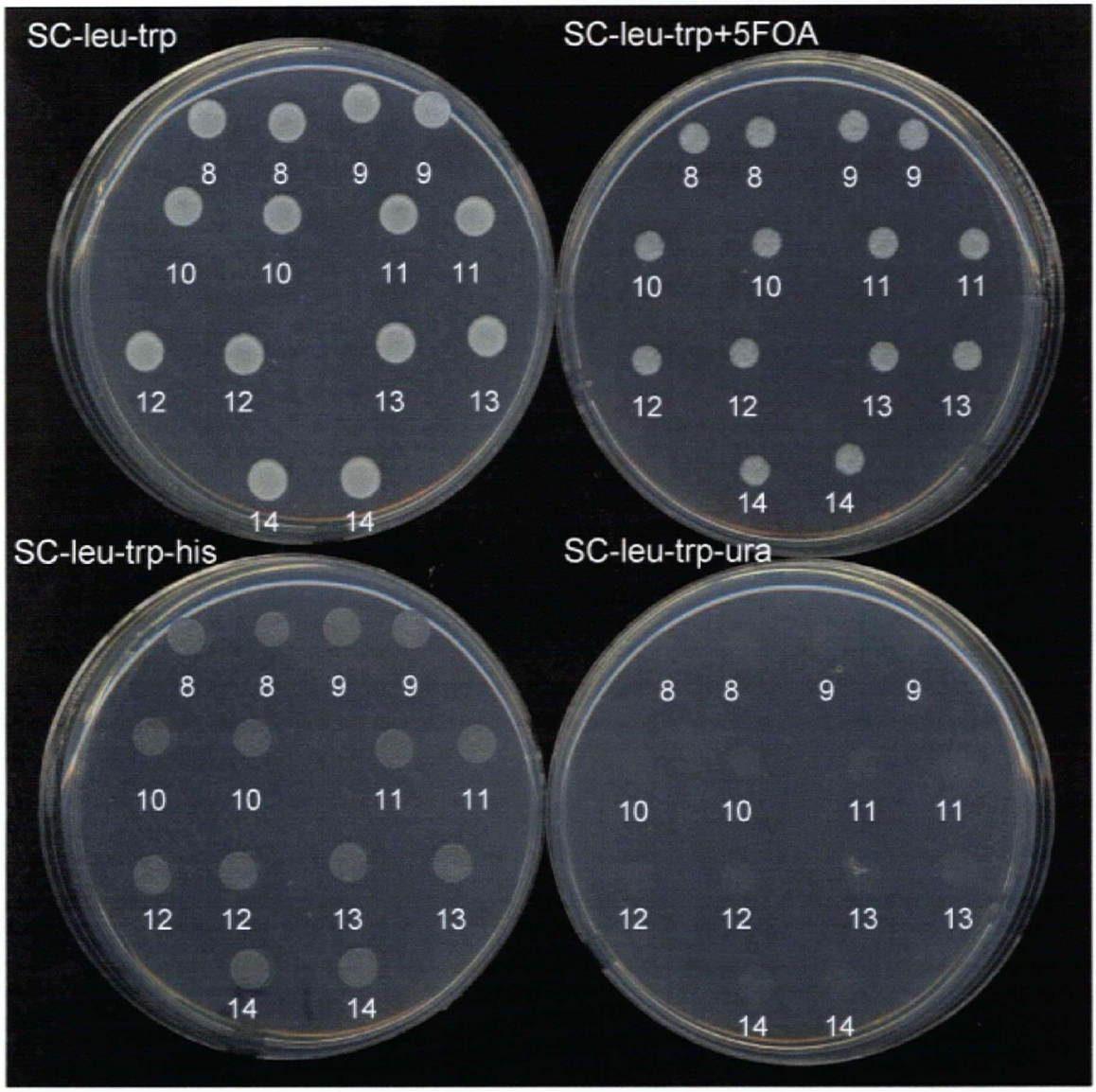

Figure 4.9. Yeast two-hybrid screening of MKK3ΔNTF2 + MPK8-14. Overnight cultures of the MaV203 strain co-transformed with MKK3ΔNTF2 and a specific MAPK (MPK8-14) grown in SC-leu-trp media were diluted 1:1 000 in PBS. Diluted cultures were spotted on duplicate agar plates containing each of four media types (SC-leu-trp, SC-leu-trp-his+3AT, SC-leu-trp-ura, SC-leu-trp+5FOA) and growth at 30°C was monitored over a 48 hour period. Growth of all combinations on SC-leu-trp media verifies yeast strains contain both bait and prey plasmids. The lack of growth of any combination on SC-leu-trp-his+3AT and SC-leu-trp-ura media types indicates the inability of each protein combination to interact. Lack of growth inhibition of all combinations on SC-leu-trp+5FOA media verifies these results.

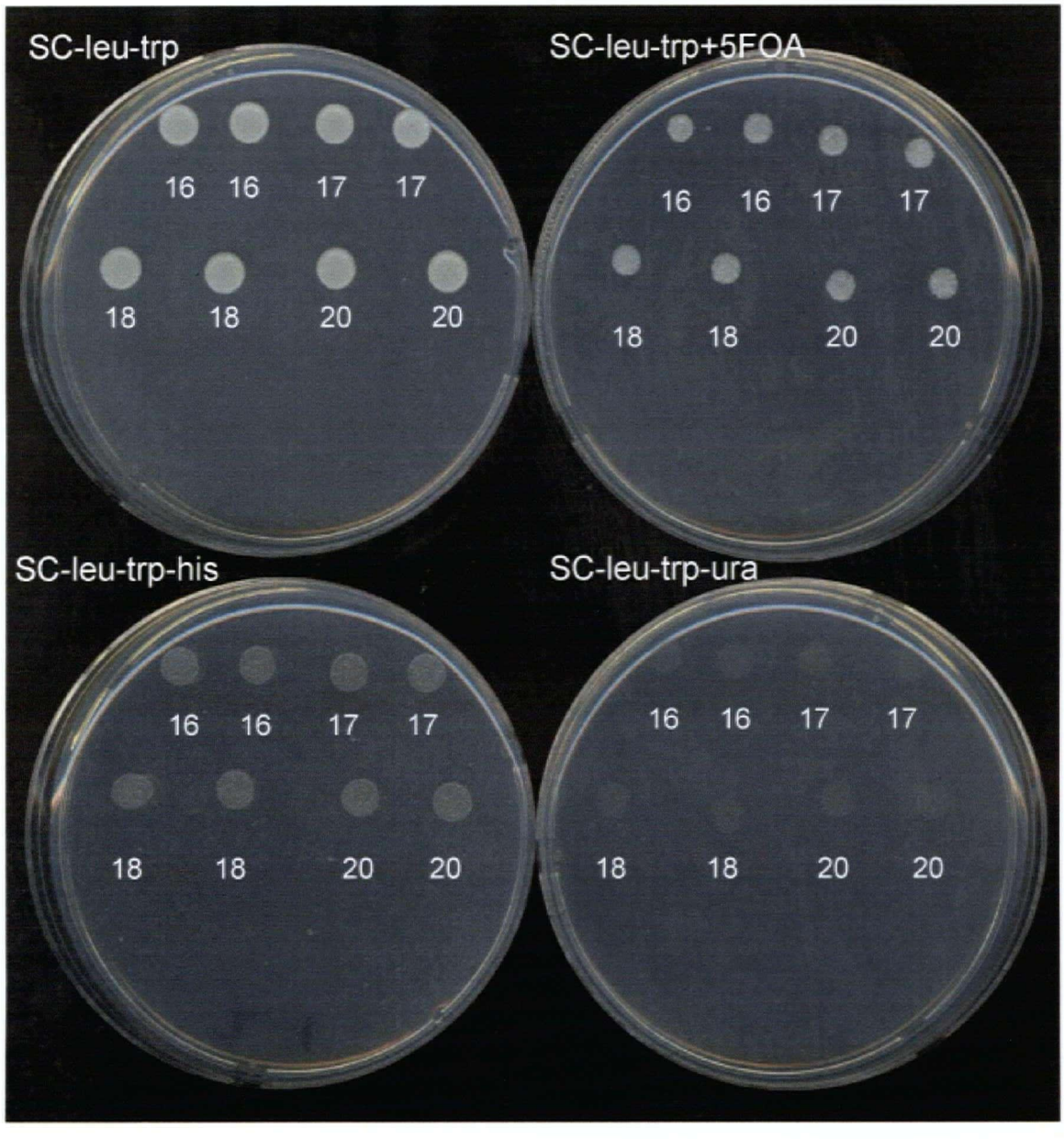

Figure 4.10. Yeast two-hybrid screening of MKK3ΔNTF2 + MPK16-20. Overnight cultures of the MaV203 strain co-transformed with MKK3ΔNTF2 and a specific MAPK (MPK16, 17 18 and 20) grown in SC-leu-trp media were diluted 1:1 000 in PBS. Diluted cultures were spotted on duplicate agar plates containing each of four media types (SC-leu-trp, SC-leu-trp-his+3AT, SC-leu-trp-ura, SC-leu-trp+5FOA) and growth at 30°C was monitored over a 48 hour period. Growth of all combinations on SC-leu-trp media verifies yeast strains contain both bait and prey plasmids. The lack of growth of any combination on SC-leu-trp-his+3AT and SC-leu-trp-ura media types indicates the inability of each protein combination to interact. Lack of growth inhibition of all combinations on SC-leu-trp+5FOA media verifies these results.

In vitro activation assays to determine substrates of MKK3 variants

In an attempt to identify the MAPK substrates of MKK3 and MKK3ΔNTF2, I assayed the ability of recombinant forms of each CA-MKK3 variant to phosphorylate, and thus activate, each of a set of 16 AtMPK proteins. Activation of the MAPK was assessed through its ability to phosphorylate the generic MAPK substrate, MBP. As a positive control for these assays, I demonstrated that CA-MKK4 activation of its known MAPK substrate, MPK6, could readily be detected on the basis of increases in the phosphorylation status of MBP (Figure 4.11). I also verified the ability of this method to detect other reported MAPKK-MAPK substrate relationships (Teige et al., 2004; Cluis, 2005), such as activation of MPK6 by CA-MKK2 and CA-MKK9, as well as the activation of MPK4 by CA-MKK2 (data not shown).

Each of full-length CA-MKK3 and the CA-MKK3ΔNTF2 variant were assayed for the ability to activate the MAPKs contained within the Ellis Research Group MAPK clone collection. On the basis of these experiments, neither MKK3 variant activated any of the MAPKs in our collection, including MPK1, MPK2 and MPK7, all of which interact with the full-length MKK3 in the yeast two-hybrid analyses. Interestingly, however, it appears that the addition of either recombinant CA-MKK3 variant to the in vitro activation assays specifically suppresses the autophosphorylation activity of MPK1, MPK2 and MPK7 (Figure 4.11). This effect was not observed for any of the remaining MAPKs in the clone set. The reaction of anti-phospho-MBP in Figure 4.11 C, lane 2 appeared strong relative to reactions containing MKK3ΔNTF2 alone or the combination of MKK3ΔNTF2 with individual MAPKs. This result was anomalous, as it was not observed in any other replicates of this activation panel.

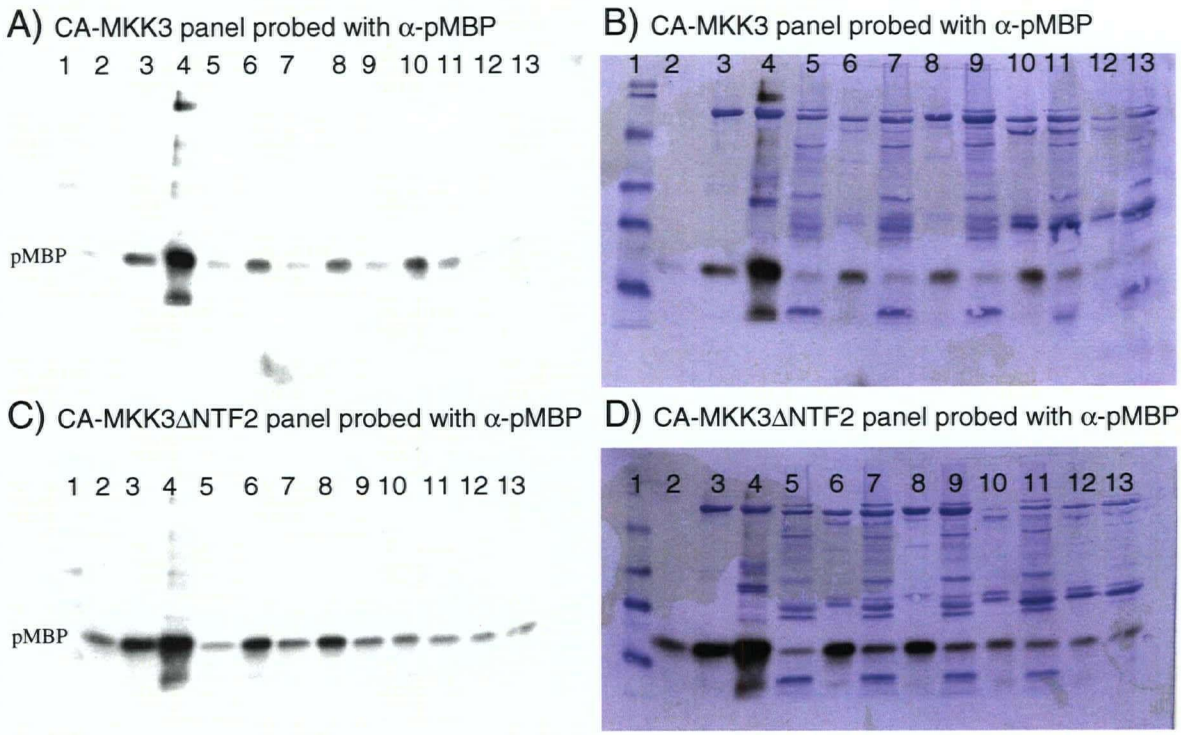

Figure 4.11. Inhibition of autophosphorylation of MKK3-interacting MAPKs. The ability of CA-MKK3 and CA-MKK3ΔNTF2 to activate group C MAPKs was examined using indirect *in vitro* activation assays that detect the phosphorylation status of a generic MAPK substrate, MBP. Co-incubation of the MKK3 derivatives (CA-MKK3 and CA-MKK3ΔNTF2) inhibits the autoactivation of MPK1, MPK2 and MPK7, as shown by reduced MBP phosphorylation (pMBP) in lanes 7, 9 and 11.

A) Immunoblot of the CA-MKK3 panel probed with anti-phospho MBP.
Lane content is as follows:

1) Protein size ladder
2) MBP only
3) MPK6
4) CA-MKK4 + MPK6
5) CA-MKK3
6) MPK1
7) CA-MKK3 + MPK1
8) MPK2
9) CA-MKK3 + MPK2
10) MPK7
11) CA-MKK3 + MPK7
12) MPK14
13) CA-MKK3 + MPK14

B) Same as A) but the immunoblot is overlaid on the PVDF membrane to illustrate equal loading of protein.

C) Immunoblot of the CA-MKK3ΔNTF2 panel probed with anti-phospho MBP. Lane content is the same as A) but using CA-MKK3ΔNTF2 instead of CA-MKK3.

D) Same as C) but the immunoblot is overlaid on the PVDF membrane to illustrate equal loading of protein.

Characterization of transgenic Arabidopsis plants carrying a dexamethasone inducible *CA-MKK3* construct

Because it is not known which biological stimuli activate MKK3, to examine the effect(s) of activating MKK3 signaling *in vivo*, transgenic Arabidopsis plants expressing a dexamethasone-inducible *CA-MKK3* variant construct were created. Because the biological impact of activation of MKK3 signaling was not known, T1 transformants were identified solely on the basis of hygromycin B resistance and allowed to grow until seed set. A collection of 10-day-old hygromycin B-resistant T2 plants were then screened for *CA-MKK3* expression induction after treatment with dexamethasone. The initial screening was conducted by RT-PCR using KK3-INF-2 and KK3-3HA-R primers which will only amplify the *CA-MKK3* message (Figure 4.12). On the basis of these results, *CA-MKK3* gene induction could be detected in lines 7, 8, 10 and 12, which were further investigated using quantitative real-time PCR. In these analyses, expression of total *MKK3* (native *MKK3* gene plus *MKK3* transgene) expression was assayed using MKK3QRT-F and MKK3QRT-R primers, and *GVG* expression levels were monitored using GVG-F and GVG-R primers (Figure 4.13).

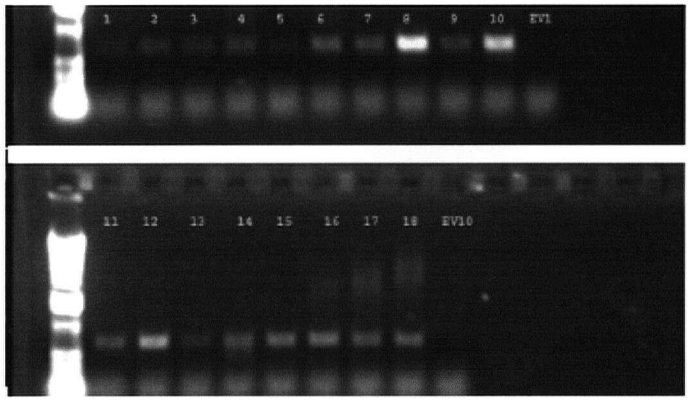

Figure 4.12. Analysis of *CA-MKK3* expression induction in transgenic dexamethasone-inducible *CA-MKK3* plants. Hygromycin B resistant T2 plants were screened for *CA-MKK3* gene induction upon treatment of 10-day old seedlings with 10 μM dexamethasone. *CA-MKK3* - gene expression induction was determined after eight hours of gene induction by PCR analysis of cDNA samples derived five representative plants from each line (transformation event) and KK3-INF2 and KK3-3HA-R primers (~700 bp amplicon). The KK3-3HA-R primer is specific to the *CA-MKK3* transcript; hence only the expression of *CA-MKK3* is represented in this analysis. Numbered lanes show the *MKK3* expression levels in the various *CA-MKK3* plant lines, while EV10 shows the *CA-MKK3* expression level in an 'empty vector' line that carries the *pTA7002* construct without a *CA-MKK3* insert.

From these analyses, it was determined that lines 7, 8 and 12 all showed *CA-MKK3* expression induction upon dexamethasone treatment. Furthermore, each of these lines showed *GVG* expression levels similar to that seen in the EV1 empty vector line (Figure 4.13), indicating that any phenotypic differences between the EV control and the CA-MKK3 lines should not be a consequence of over-expression of GVG. However, I decided against using CA-MKK3 line 10 in further experiments since its *GVG* expression levels were even higher than those in the EV10 empty vector line, and the latter has been found to display a moderate dexamethasone-induced phenotype (data not shown).

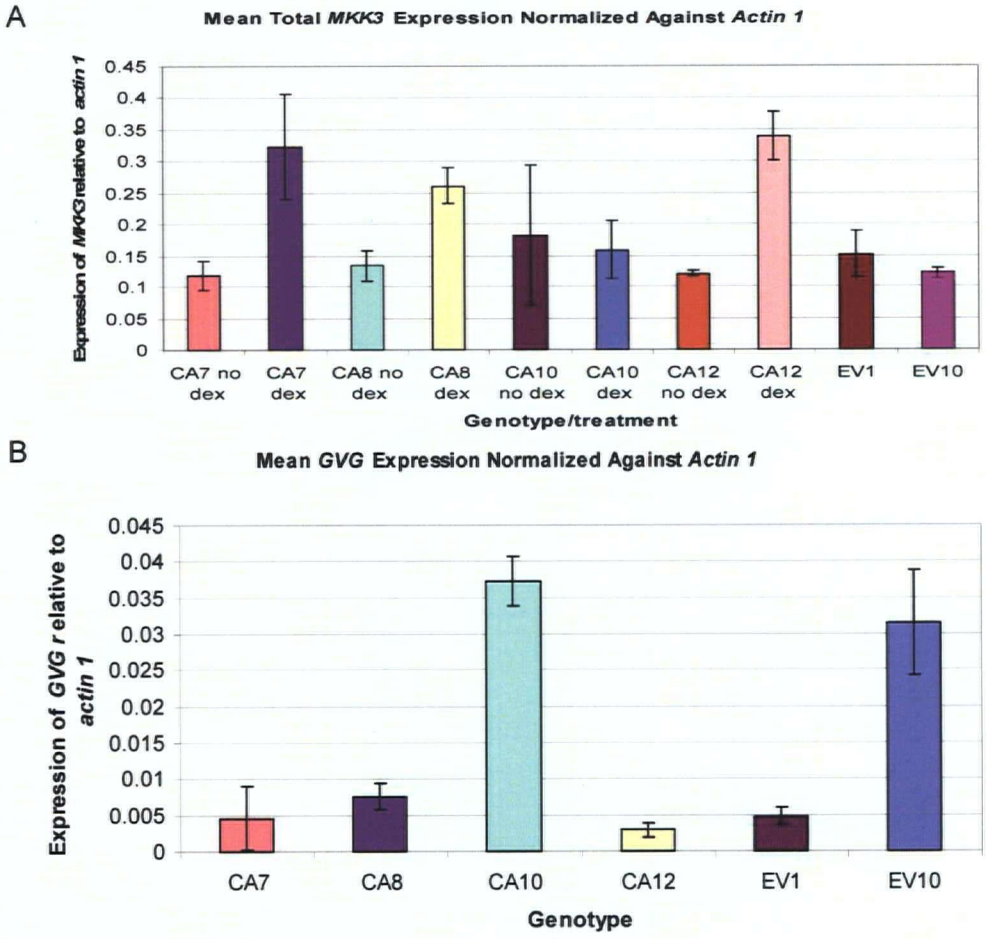

Figure 4.13. Mean total *MKK3* and *GVG* gene expression analysis in transgenic dexamethasone-inducible *CA-MKK3* plants. Ten-day-old seedlings were treated with 10 μM dexamethasone and gene expression was quantified using real-time PCR following eight hours of induction. Total *MKK3* expression (A) was evaluated using MKK3QRT-F and MKK3QRT-R primers and *GVG* expression (B) was quantified using GVG-F and GVG-R primers.

Phenotypic analysis of *CA-MKK3* induction

In an attempt to decipher the biological outcome of activation MKK3 signaling *in vivo*, the transgenic Arabidopsis plants carrying the dexamethasone-inducible *CA-MKK3* variant were treated with dexamethasone at various time points throughout development and monitored for the presence of any MKK3-induced phenotypes (Table 4.1). Similar analyses were conducted

with seedlings grown in tissue culture on ½ MS agar plates (Table 4.1). No visible differences were detected following *CA-MKK3* expression induction in any of the experiments.

Table 4.1. Phenotypic analysis of CA-MKK3 gene induction. Transgenic Arabidopsis plants carrying a dexamethasone-inducible CA-MKK3 construct were treated as described to induce CA-MKK3 expression and monitored for the development of abnormal phenotypes.

Treatment Stage	Treatment Type/Growth Medium	Outcome
Germinating seeds	½ MS + 10 μM dexamethasone	No difference in germination rates between mock-treated and dexamethasone-treated seeds in any CA-MKK3 line or empty-vector line
3-day old seedlings	3-day old seedlings transferred to ½ MS + 10 μM dexamethasone	No difference in growth (aerial portions or roots) between mock-treated and dexamethasone-treated plants in any CA-MKK3 line or empty-vector line
10-day old seedlings	10-day old seedlings transferred to ½ MS + 10 μM dexamethasone	No difference in growth, leaf appearance or root appearance between mock-treated and dexamethasone-treated plants in any CA-KK3 line or empty-vector line
14-day old seedlings	14-day old seedlings transferred to ½ MS + 10 μM dexamethasone	No difference in growth, leaf appearance or root appearance, including lateral roots, between mock-treated and dexamethasone-treated plants in any CA-MKK3 line or empty-vector line
21-day old plants	21-day old pre-bolting plants grown on Redi-earth sprayed with 25 μM dexamethasone	No difference in growth denoted by leaf morphology and time until bolting between mock-treated and dexamethasone-treated plants in any CA-MKK3 line or empty-vector line
28-day old plants	28-day old flowering plants grown on Redi-earth sprayed with 25 μM dexamethasone	No difference in growth, in flower morphology or in silique development between mock-treated and dexamethasone-treated plants in any CA-MKK3 line or empty-vector line
35-day old plants	35-day old flowering plants grown on Redi-earth sprayed with 25 μM dexamethasone	No difference in seed set between mock-treated and dexamethasone-treated plants in any CA-MKK3 line or empty-vector line

Gene expression profiling of dexamethasone-induced CA-MKK3 signaling

Due to the lack of a visible phenotype associated with the induction of MKK3 signaling in the dexamethasone-inducible CA-MKK3 transgenic Arabidopsis lines, a microarray-based gene

expression profiling experiment was designed to assess the transcriptional response of the transgenic plants to activation of MKK3 signaling. Dexamethasone-induced CA-MKK3 lines were to be compared with mock-treated CA-MKK3 lines five hours following *CA-MKK3* gene induction using two biological and two technical (dye swap) replicates. Each biological replicate comprised tissue derived from twenty 21-day-old pre-bolting plants. Twenty-one-day-old pre-bolting plants were either treated with 25 μM dexamethasone solution, or mock-treated, by spraying plants to run-off. Prior to large-scale RNA isolation, the level of *CA-MKK3* gene induction was surveyed in samples derived from each replicate, using real-time PCR (Figure 4.14). Consistent *CA-MKK3* gene induction was not detected, nor could it be detected in a similar experiment using cDNA derived from pooled tissue of ten dexamethasone-treated plants in a subsequent study (data not shown). Due to my inability to achieve consistent dexamethasone induction in large pools of the *CA-MKK3* transgenic plants, this expression profiling experiment was not completed.

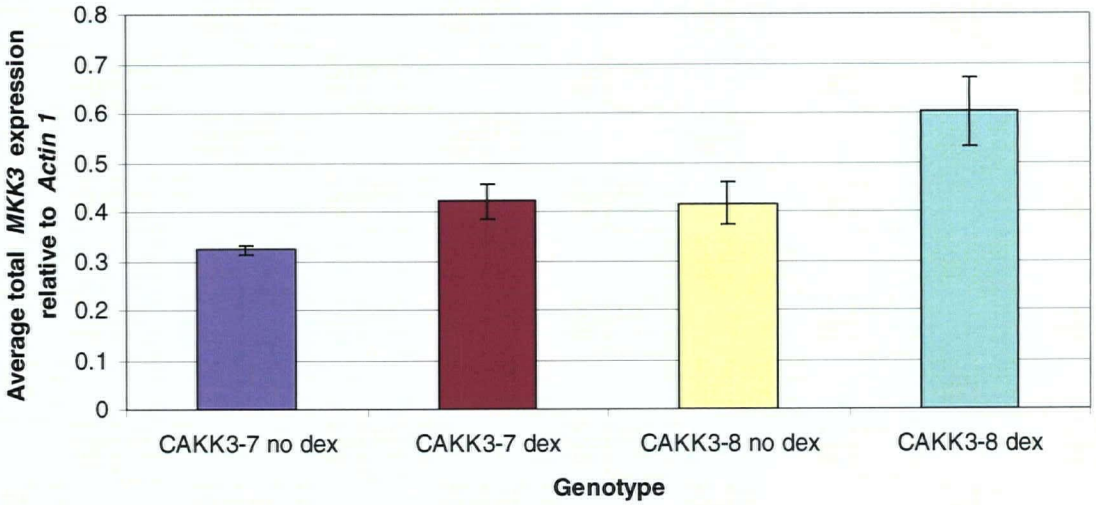

Figure 4.14. Quantification of MKK3 expression in tissue samples designated for CA-MKK3-induced gene expression profiling experiments. Average total expression of *MKK3* relative to *Actin 1* was determined by real-time PCR using template cDNA samples generated from representative samples of tissue prepared for microarray analysis.

DISCUSSION

Yeast two-hybrid interactions of MKK3 variants with MAPKs

The presence of a C-terminal NTF2 domain in plant group B MAPKKs such as AtMKK3 is intriguing since, to date, no other MAPKKs have been found to share this protein architecture. However, three-component MAPK signaling modules are present in all eukaryotes, including plants, which implies that the fusion between an ancestral MAPKK and the NTF2 domain occurred uniquely in photosynthetic organisms. Furthermore, the fact that a group B MAPKK is encoded by the genome of the unicellular photosynthetic alga, *Chlamydomonas* (Hamel et al., 2006), also indicates that this domain fusion occurred prior to the evolution of multicellular, photosynthetic organisms. This suggests that whatever signaling modules MKK3 may be involved in are likely to participate in core biological processes that were already operative in the unicellular ancestors of modern plants. My characterization of MKK3 function has indicated that MKK3 signaling may be involved in the plant response to certain stresses and to particular phytohormones (Chapter 2; Chapter 3), and in plant development. However, the role of the NTF2 domain in MKK3 remains elusive. The yeast two-hybrid analyses revealed that the NTF2 domain is required for the interaction of MKK3 with three specific MAPKs, MPK1, MPK2 and MPK7, and that elimination of the NTF2 domain in the MKK3ΔNTF2 variant suppresses these interactions (Figure 4.8). Although false-positive interactions have been a common feature of yeast two-hybrid screens (Bartel et al., 1993; Vidalain et al., 2004), the ProQuest Yeast Two-hybrid system used in my study employs a number of techniques to reduce this phenomenon (Invitrogen, Burlington, ON, Canada). First, the ProQuest system uses low copy number bait and prey plasmids, which has been shown to significant reduce the occurrence of false positives

(Vidalain et al., 2004). Furthermore, use of the MaV203 yeast strain allows yeast two-hybrid interactions to be screened with simultaneous use of four reporter systems (histidine auxotrophy, uracil auxotrophy, FOA-inhibition and *beta*-galactosidase activity), each of which uses discrete promoters. Since my goal was to identify which MAPKs the MKK3 variants might interact with, rather than quantifying the strength nature of the interactions, only the first three screening methods were employed. Verification that MKK3 indeed interacts more strongly with MPK7 than with the other two MAPKs could be accomplished using the *beta*-galactosidase reporter system. This work is currently being carried out in the Ellis Laboratory.

Arabidopsis MPK1, MPK2, MPK7 and MPK14 all belong to the phylogenetically-related group C MAPKs (Ichimura et al., 2002), and share a high degree of sequence similarity. However, there was no indication in the yeast two-hybrid assays that MPK14 could interact with MKK3 Alignment of group C MAPK amino acid sequences reveals two candidate regions that might allow MKK3 to distinguish between the three positive interactors (MPK1, MPK2, MPK7) and MPK14 (Figure 4.15). MPK1, MPK2 and MPK7 each contain a three amino acid insertion (-SXX-) located five amino acids upstream of the -TEY- activation motif, whereas this insertion is absent from the MPK14 sequence. Of the remaining -TEY- containing MAPKs, only MPK13 contains a somewhat related insertion (-SX-) at this location (-SX-; Appendix 6). Interestingly, each of the group D -TDY- containing MAPKs contains the -SXX- insertion, but they also contain an additional three amino acid insertion upstream of the -TDY- motif. Since none of the group D MAPKs interacted with MKK3 in the yeast two-hybrid screen, perhaps this additional insertion could be preventing MKK3 from interacting with this site (Appendix 6). Alternatively, the presence of a

-TDY- motif and not a -TEY- motif in these kinases may prevent the interaction.

Figure 4.15. Multiple sequence alignment of Arabidopsis group C MAPKs. Alignments were performed using ClustalW version 1.83. -TEY- motifs of each MAPK are highlighted (yellow). Potential points of interaction between MKK3 and each of MPK1, (green) MPK2 (green) and MPK7 (cyan) are also highlighted.

A similar pattern can be seen closer to the C-terminus of each of these MAPKs. MPK14 contains a serine insertion in a conserved -PIXLD- motif, which creates the variant -PVSSLE- motif (Figure 4.15). However, all other -TEY- MAPKs and all -TDY- MAPKs encode variants of this motif that include either insertions or deletions (shown in Appendix 6). These two small regions appear to be the most likely sites at which MKK3 could discriminate between MPK1, MPK2 and MPK7 and other MAPKs, but more detailed analysis using sequence variants mutagenized in these regions would be needed to test this hypothesis.

Because the MKK3ΔNTF2 variant cannot interact with these three MAPK proteins, the ability of the NTF2 domain alone to interact with these proteins also needs to be tested.

In vitro identification of MKK3 substrates

The data from the yeast two-hybrid interaction studies and the *in vitro* activation panel did not overlap, since full-length recombinant CA-MKK3 does not appear to activate MPK1, MPK2 or MPK7, nor did it activate any other MAPK in the test collection. This collection lacked MPK8, MPK15, MPK18 and MPK20, due to problems producing recombinant versions of these proteins in *E. coli*, so it is still possible that one or more of these "missing" MAPKs can act as a substrate of CA-MKK3. Production of these recombinant proteins using a different system, such as yeast expression, or a cell-free *in vitro transcription/translation* expression system would allow this phosphorylation screen to be completed.

Curiously, MKK3 did appear to maintain some ability to interact with MPK1, MPK2 and MPK7 since addition of CA-MKK3 to the *in vitro* activation assays inhibited the autophosphorylation (autoactivation) of each of these kinases. In this case, however, incubation of the MKK3ΔNTF2 variant with each of these MAPKs also prevents autoactivation. Once again, this phenomenon was not observed for MPK14 (Figure 4.11), nor was it seen for any of the other MAPKs included in the panel (data not shown). If the interaction between MKK3 and these MAPKs occurs through the -SXX- motif that lies immediately upstream of the -TEY- motif, as discussed above, the ability of MKK3 to prevent autophosphorylation of these MAPKs may result from steric hindrance that physically blocks MAPK access to the target -TEY- motif.

The failure to detect the activation of any recombinant MAPK by either of the MKK3 variants could be the result(s) of several factors. First, although there has been no report of a MAPKK that is not made catalytically active upon modification of the -S/TXXXXXS/T- motif to

-E/DXXXXXE/D-, it is always possible that modification of MKK3 in this fashion does fail to activate the kinase. Although several potential generic substrates have been tested for their ability to be phosphorylated by active MAPKKs, including -TXY- containing peptide fragments, no generic MAPKK substrate has yet been identified (Seger et al., 1992). In the absence of such a positive control, I am unable to definitively demonstrate catalytic activity of either MKK3 variant.

The possibility also exists that I did not detect MKK3 substrates in my indirect reporter system because not all activated MAPKs are able to utilize MBP as a substrate. However, there have been no reports of MAPKs failing to use MBP as a substrate *in vitro*, and all plant MAPKs tested to date, including both -TEY- and -TDY- containing MAPKs, have been shown to use MBP as a substrate (Samuel and Ellis, 2002; Cheong et al., 2003). Furthermore, all MAPKs used in this panel displayed at least some level of autoactivation as shown by phosphorylation of MBP in my indirect reporter system.

The remaining possibility is that the activation of one or more MAPK by MKK3 occurred but did so at a level that was below the threshold of detection of the anti-phospho-MBP antibody. Such an outcome could have been due to detection limits of the antibody, or more likely, due to my inability to distinguish between MAPK autoactivation and MKK3-induced activation. Since all the MAPKs tested displayed some level of autoactivation and phosphorylation of MBP, a more discriminating assay would require the use of kinase-inactive variants of each recombinant MAPK, and detection of phosphorylation using ^{32}P-labeled ATP. Phosphorylation of a MAPK by MKK3 using this approach does not demonstrate activation of the MAPK, but at least it might enable me to define the subset of Arabidopsis MAPKs that can serve as MKK3 substrates.

It is important to recognize that non-catalytic interactions between plant MAPKKs and MAPKs have also been reported. The tobacco MAPKK, SIPKK, was initially identified on the basis of a physical interaction with SIPK (Liu et al., 2000), but subsequent *in vitro* phosphorylation studies revealed that SIPK is not a substrate of SIPKK. Instead, the MAPK, NtMPK4, was found to be activated by SIPKK (Gomi et al., 2005). Thus, the results of my experiments can be interpreted in various ways. For example, the apparent contradiction between MKK3 interacting with, but not activating, specific MAPKs may actually reflect a regulatory mechanism in which MKK3 functions to inhibit the activation of these three MAPKs by sequestering them in an inactive form in the cell.

Little is known regarding the function of the group C MAPKs. MPK1 and MPK2 were initially identified as auxin-responsive gene products, and exposure of Arabidopsis plants to exogenous auxin was shown to induce rapid activation of a protein kinase activity that was capable of activating recombinant MPK2 *in vitro* (Mizoguchi et al., 1994), although the identity of the activating kinase was not established. Furthermore, both *MPK1* and *MPK2* were shown to be expressed in all plant tissues except mature seeds, which correlates with the expression pattern of *MKK3*. Publicly available microarray datasets agree with these results (Genevestigator). Mizoguchi et al (1994) also observed activation of both recombinant MPK1 and MPK2 by recombinant *Xenopus* MAPKK, using an in vitro MBP phosphorylation assay to detect the activity of MPK1 and MPK2. The sequence of the *Xenopus* MAPKK used in this study is most similar to that of AtMKK1, and shows weaker similarity to AtMKK2, AtMKK6, AtMKK8 and finally AtMKK3. Therefore, while these studies show that Arabidopsis MPK1 and MPK2 can serve as substrates of MAPKKs, that they can phosphorylate MBP when activated, they do not

immediately suggest, at least on the basis of sequence homology between eukaryotic MAPKKs, that these MAPKs are MKK3 substrates.

MKK1 has been reported to interact with MPK1 in yeast two-hybrid studies suggesting that, while MKK3 appears to prevent autoactivation of MPK1, MKK1 may be the "true" upstream kinase activating MPK1 (Ichimura et al., 1998). The study by Ichimura et al (1998) did not include MPK2 or MPK7.

Analysis of publicly available microarray data for expression profiles of *MPK7* illustrates that it is expressed at slightly higher levels than MKK3, but in a similar overall pattern to *MKK3*, including up-regulation (~2-fold) in response to both salt and ABA treatments (Genevestigator). As described previously (Chapter 2), both *MKK3* and *MPK7* encode predicted targets of miRNA, consistent with the idea that the expression of these kinases may be co-regulated. Analysis of *mpk7* loss-of-function or over-expression plants would provide more insight into the link between these kinases, but no T-DNA insertion lines are available at this locus, perhaps indicating the *MPK7* is an essential gene.

The only other functional data reported for group C MAPKs involved studies from other plant species. It has been reported that *OsMAPK4* expression can be induced by multiple environmental stresses including sugar starvation, cold and salt stress (Fu et al., 2002). OsMAPK4 has recently be re-annotated as OsMPK7, based on phylogenetic analysis (Hamel et al., 2006), and the induction behaviour reported above may indicate that group C MAPKs are involved in stress responses (Fu et al., 2002). A role for group C MAPKs in development has also been suggested, since the pattern of *NtF3* expression in tobacco has been associated with anther development (Wilson et al., 1993), and petunia *PMEK1* expression is responsive to auxin and may function in the regulation of the cell cycle (Trehin et al., 1998). Thus, although clear

functional data is lacking, as it is for MKK3, group C MAPKs do appear to be involved in several aspects of development and stress responses.

The overlapping expression patterns for these kinases, coupled with the apparent inhibition of activity of the MAPKs, MPK1, MPK2 and MPK7, by catalytically active MKK3 suggests that, while each of these kinases is involved in various aspects of development and stress responses, they may be functionally related at some level. If MKK3 truly functions as a negative regulator of development, perhaps MAPK signaling modules including MPK1, MPK2 and MPK7 act to promote development. Identification of the upstream activators of these MAPKs, and phenotypic analysis of over-expression and loss-of-function mutants, should help resolve this question.

The observation that the MKK3ΔNTF2 variant was unable to interact with any of the MAPKs in the yeast two-hybrid analysis, but could interact with MPK1, MPK2 and MPK7 in the *in vitro* activation assays, could indicate that the NTF2 domain is crucial for protein interactions *in vivo* but not *in vitro*. The ability of the MKK3ΔNTF2 variant to interact differently with these proteins in different contexts could be due to multiple interaction domains in MKK3. A characteristic feature of MAPKKs is the presence of an N-terminal docking domain that is often required for the interaction of MAPKK with cognate MAPK substrates (Kiegerl et al., 2000; Ichimura et al., 2002). While this has not been systematically investigated in the plant MAPKKs, deletion of this docking domain from the alfalfa MAPKK, SIMKK, did not completely abolish the ability of SIMKK to interact with its cognate MAPK, SIMK, suggesting that additional interaction sites must be present (Kiegerl et al., 2000). NTF2 proteins and NTF2 domain-containing proteins exist *in vivo* as dimers, either as homodimers in the case of NTF2 (Chaillan-Huntington et al., 2001) or heterodimers in the case of NTF2 domain-containing

proteins such as Mex67 (Thakurta et al., 2004). It is tempting to speculate that, in the purified protein samples used for the *in vitro* activation assays, the interaction between MKK3 and these MAPKs (via the docking domain) may have been sufficiently strong to prevent autophosphorylation of the MAPKs, whereas in the milieu of the yeast cell this interaction is not sufficient, and either an interacting full-length MKK3 protein or another cofactor is required to confer a stable interaction. Similarly, since we currently know little about the composition of putative MAPK signaling complexes in plants, additional cofactors may be required for MAPKK phosphorylation of cognate MAPKs. Direct measurement of *in vivo* MAPK activation resulting from activation of MKK3 *in planta* might clarify this.

Analysis of transgenic plants expressing a CA-MKK3 variant

Although several stimuli are known to induce *MKK3* gene expression, stimuli resulting in the phosphorylation of MKK3, and hence, activation of MKK3 signaling modules, remain to be identified. I therefore attempted to study the effect of directly inducing MKK3 activity in transgenic Arabidopsis plants by expressing a dexamethasone-inducible *CA-MKK3* construct. Repeated induction experiments illustrated that *CA-MKK3* expression was induced and could be detected following dexamethasone treatment. Prior to phenotypic analysis of the *CA-MKK3* transgenic plants, *GVG* expression was analysed in these lines for two reasons: First, very high levels of *GVG* expression have been reported to be correlated with tissue damage when *pTA7002* empty vector plants were treated with dexamethasone (Kang et al., 1999; Andersen et al., 2003). Thus, lines possessing low *GVG* are essential for phenotypic characterization studies. *GVG* expression also had to be quantified in order to select the appropriate empty vector control line to be used in comparison experiments such as microarray profiling.

Initially, microarray profiling studies were to be conducted to determine the transcriptional impacts of activating MKK3 signaling. Large quantities of RNA (~160 μg per biological replicate) are required for these studies, which necessitated pooling tissue derived from 15-20 Arabidopsis plants. However, analysis of *MKK3* expression in cDNA derived from pooled dexamethasone-treated tissue samples showed that the mean *MKK3* expression levels were, at best, marginally different from untreated samples. This likely reflects non-uniform induction of gene expression, perhaps related to the delivery of dexamethasone by spraying plants, but in any event, the planned microarray studies were not completed. However, such expression profiling studies should be informative and could perhaps be conducted in the future using RNA derived from single plants and RNA signal amplification techniques such as the "3DNA" dendrimer probe system (Genisphere, Hatfield, PA).

Induction of *CA-MKK3* expression at several time points throughout development ultimately did not reveal any phenotypic consequences of such expression. In light of my inability to detect activity of CA-MKK3 *in vitro* these results could potentially be explained by a lack of catalytic activity on the part of the expressed CA-MKK3. At this point, I have no way of resolving that question. The *CA-MKK3* expression induction observed, even in the highest expressing lines, was only two- to three-fold above endogenous levels of *MKK3*, which is lower than I had expected, based on similar studies also using dexamethasone-inducible MAPKKs. Dexamethasone induction of *CA-MKK4* and *CA-MKK9*, for example, results in increased expression of five- to ten-fold (personal communication, Dr. M. Samuel; Cluis, 2005). It is possible that induction to the modest levels attained with *CA-MKK3* is not high enough to generate phenotypic effects. Finally, as is generally the case with gene expression studies, the

degree to which *CA-MKK3* gene expression and accumulated CA-MKK3 protein levels in the cell are directly correlated is unknown.

Perhaps the greatest influence on the lack of a detectable phenotype in these lines results from the dexamethasone-inducible system itself. The presence of high levels of the GVG transcription factor can impair plant development in the presence of dexamethasone (Kang et al., 1999; Andersen et al., 2003), and the phenotypes of such plants resemble mutants with defective ethylene and auxin signaling pathways (Kang et al., 1999; Andersen et al., 2003). Although these earlier studies examined the effects of high levels of *GVG* expression, prolonged exposure to dexamethasone has deleterious effects on growth of even the plants expressing the lowest levels of *GVG* in this study. Since the current hypothesis regarding MKK3 signaling is that it functions as a negative regulator of development and appears to be involved in signaling pathways involving auxin (Chapter 2; Chapter 3) and perhaps ethylene (Chapter 3), perturbed development due to *GVG* expression may have hindered my ability to detect a more subtle MKK3-induced phenotype. Analysis of induced MKK3 activity using a different inducible promoter system, such as the oestrogen-, alcohol- or tetracycline-inducible systems (Moore et al., 2006) might make it possible to overcome this putative limitation.

CONCLUSIONS

Data reported in Chapters 2 and 3 suggest that MKK3 functions in development, stress- and phytohormone-responses. The attempt to examine the phenotypic effect of inducing MKK3 activity using a dexamethasone inducible, constitutively active variant of MKK3 (CA-MKK3) proved to be ineffective and should be further examined *in vivo* using a different inducible promoter system that allows long-term gene expression induction.

Nonetheless, biochemical identification of the complete collection of interacting proteins and substrates of MAPKKs will provide insight into both the biological functions of MAPK signaling modules and how these modules are regulated. The yeast two-hybrid analyses completed in this study revealed that MKK3 is able to interact with some, but not all of the group C MAPKs. Specifically, MKK3 was able to interact with MPK1, MPK2 and MPK7 in an NTF2 domain-dependent fashion in yeast two-hybrid assays. Indirect activation studies using a constitutively active variant of MKK3 (CA-MKK3) revealed that these interacting MAPKs do not appear to be substrates of MKK3. Rather, it appears that the interaction with MKK3 may serve a regulatory function in which MKK3 prevents autoactivation of each of MPK1, MPK2 and MPK7. While no aberrant phenotype has been identified in *mkk3*-null or MKK3 over-expression plants, future characterization of the biological functions of MAPK signaling involving group C MAPKs may provide greater details regarding the role of MKK3 in these processes.

CHAPTER 5. General discussion

Studying MAPK signaling modules using reverse-genetics approaches

There are approximately 90 genes encoding members of the MAPKKK (60), MAPKK (10) and MAPK (20) gene families in Arabidopsis (Ichimura et al., 2002; Hamel et al., 2006) but functional information exists for only a few of these kinases. Extensive forward genetic screens have been conducted to identify mutants displaying altered sensitivities to phytohormones, diminished capacities to tolerate biotic and abiotic stresses and modified developmental patterns, and in a few cases these screens have retrieved specific MAPK signaling module components. For example, *mkk7*-null mutants show increased polar auxin transport (Dai et al., 2006), *mpk4*-null mutants are dwarfed and have a defective jasmonate response (Petersen et al., 2000) and *yda*-loss-of-function mutants produce excessive numbers of stomata as well as showing embryo defects (Bergmann et al., 2004). Direct biochemical analyses have revealed that several other MAPK module components function in various facets of plant physiology (Ichimura et al., 2000; Mockaitis and Howell, 2000; Samuel et al., 2000; Fu et al., 2002; Cheong et al., 2003; Kim et al., 2003). However, the functions of most members of all three module families have yet to be discovered through these approaches, which raises the question of how we can identify the functions of the uncharacterized MAPK signaling components? In terms of currently available technologies, the answer to this question usually involves the application of reverse genetics approaches, such as those employed in my research program. However, while these methods are powerful, and can provide valuable insights into the biological function of a specific protein,

they are by no means fail-safe, particularly when gain-of-function or loss-of-function mutants fail to display a detectable, abnormal phenotype - the "holy grail" of plant genetics.

A general approach to characterizing genes of unknown function involves expression profiling to gain insight into associations between gene expression and cellular/tissue function. In addition, loss-of-function and over-expression genotypes will be characterized to varying degrees. If the encoded protein displays informative structural features, it may be worth generating plant lines expressing variants of the native structure, in order to try to link putative protein functionality to phenotype. Sub-cellular localization studies using fluorescently tagged versions of the encoded protein can be useful since cellular localization patterns can provide important insights into biological function. However, all of these experimental approaches are predicated on the assumption that the function(s) in question will be revealed in a developmental or performance phenotype once the gene has been appropriately manipulated. Unfortunately, phenotypic analysis of single gene loss-of-function mutants often proves ineffective, primarily for two critical reasons. First, extensive functional redundancy may exist amongst gene family members, which means that loss of a single family member can often be compensated for by partial functional contributions from other members. Second, because signaling molecules such as MAPKKs may perform highly specialized functions, the usual phenotype characterization panels could lack the necessary resolution to identify subtle phenotypes or phenotypes that manifest themselves at a biochemical (e.g. altered hormone or secondary metabolite production) or microscopic level (e.g. altered cell structure). However, in the absence of an initial phenotype to pursue, complete systematic testing for all possible conditional phenotypes is not a realistic goal for most laboratories.

Over-expression of a particular gene is one useful strategy for overcoming the problem of family member redundancy. Depending upon the function of the encoded protein, disruption of the stoichiometric balance of that protein within the cell may have informative phenotypic consequences. However, these consequences may be masked by additional regulatory mechanisms, such as a requirement for post-translational activation, as is likely the case for many MAPK module components. Furthermore, the correlation between over-expression of a gene and over-accumulation of the encoded protein is specific to each gene. It is becoming clear that the regulation of expression of critical genes, such as those encoding proteins involved in development or hormone signaling, occurs at several levels, including transcriptional, post-transcriptional, translational, and post-translational control points. Because we are only beginning to characterize and understand some of these forms of regulation, the ability to over-express any particular gene must be tested empirically.

Thus, despite the power of these reverse genetics approaches, failure to identify biological functions for genes using classical reverse genetics strategies appears to be not uncommon (Bouche and Bouchez, 2001), and it is clear that additional experimental methods will be required to functionally characterize the 26,000+ genes that comprise the Arabidopsis genome. The most promising avenues currently available are global analyses such as transcriptional profiling, metabolic profiling and protein profiling, all of which can be integrated into what has come to be known as a 'systems biology' approach. Because global approaches such as these ultimately serve as hypothesis-generating experiments, inferred biological functions must still be verified using additional techniques. In the context of MAPK signaling modules, profiling data will allow the design of *in vivo* experiments aimed at analyzing either the effects of altering putative up- and down-stream signaling components that interact with MAPK modules, or the

effect of generating combinatorial loss-of-function mutants for members of each gene family. Because the canonical components of MAPK signaling modules (MAPKKK, MAPKK and MAPK) lie upstream of the biological effector proteins such as transcription factors, biological characterization of each component may require experiments of this nature. Furthermore, continued identification of inducible, tissue- specific promoter systems to facilitate the expression of genes encoding variant proteins will serve to complement these studies by providing a means to ascertain highly specific, context-dependent biological functions for each gene. Finally, the data provided both by global approaches and by analysis of combinatorial loss-of-function mutants should also provide starting points to recreate signaling pathways *in vitro* using biochemical analyses such as those reported in this thesis.

MKK3 signaling in relation to other MAPKs

Knowledge pertaining to MAPK signaling in Arabidopsis is rapidly expanding and reports to date indicate that multiple MAPK signaling modules are involved in phytohormone, stress and developmental signaling (Ichimura et al., 2002; Tanoue and Nishida, 2003; Pedley and Martin, 2005; Hamel et al., 2006). The research reported in this thesis adds to this knowledge base (Figure 5.1) by providing much needed information regarding MKK3 signaling. Thus, we now have functional information pertaining to each functionally relevant Arabidopsis MAPKK (both MKK8 and MKK10 have been suggested to be non-functional MAPKKs (Hamel et al., 2006)).

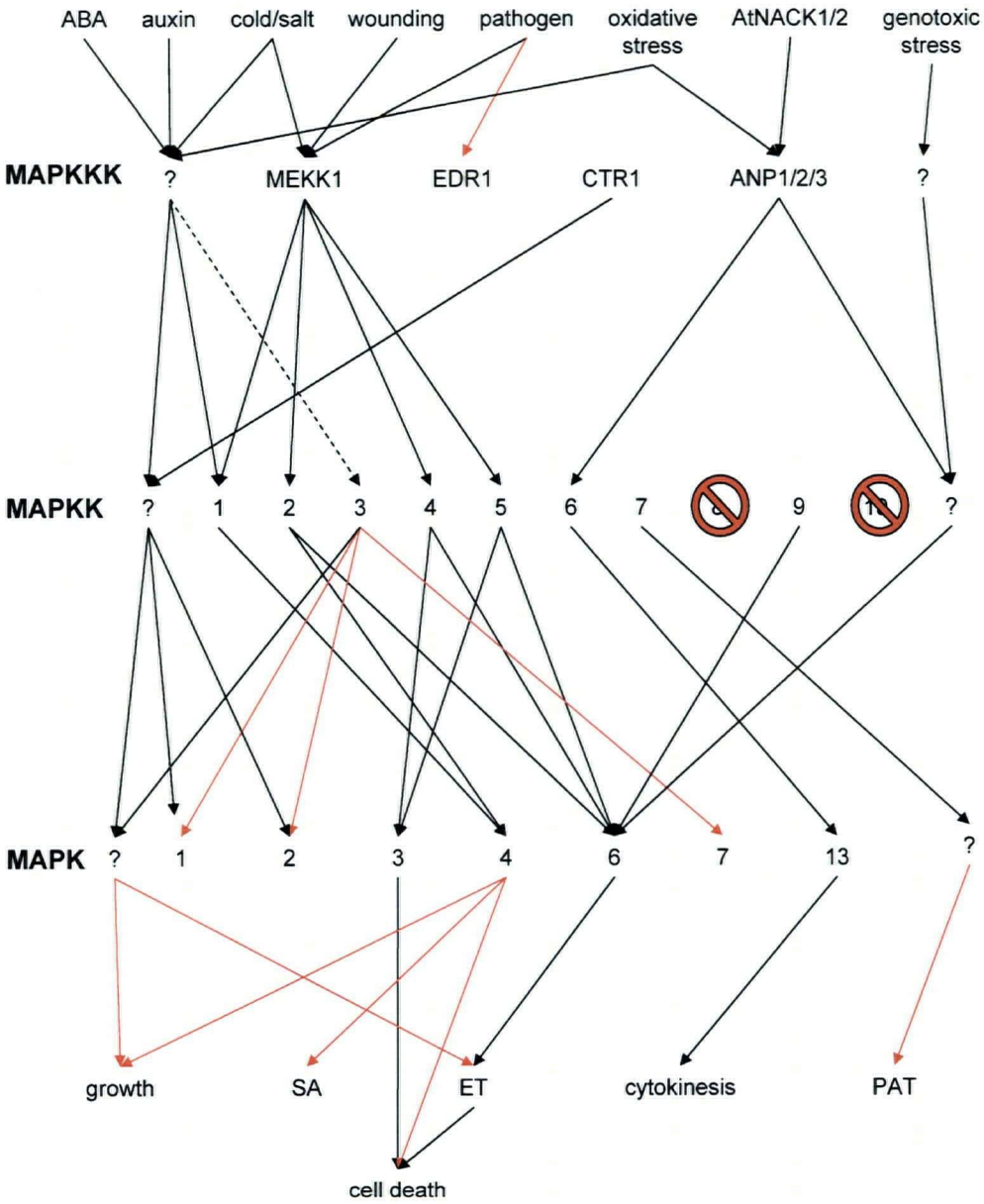

Figure 5.1. Schematic representation of MAPK signaling modules in Arabidopsis. Arabidopsis MAPK signaling modules have roles in development, and responses to both environmental stresses and phytohormones. Positive, or activation reactions are illustrated in black. Transcriptional up-regulation of MKK3 is illustrated by a dashed arrow. Inhibitory interactions are depicted in red. Expression and structural analysis suggests that MKK8 and MKK10 may not be biologically active.

It appears that MKK3 signaling is involved in several facets of plant physiology, including development, phytohormone and stress responses. Specifically, it appears that MKK3 may function as a negative regulator of plant growth in response to phytohormones and environmental stresses. A reported function of auxin in floral development is to inhibit the

growth/development of tissues in close proximity to high concentrations of auxin. The correlation between *MKK3* and auxin accumulation in these tissues, coupled with the suggestion that MKK3 functions as a negative regulator of growth in response to phytohormones, including auxin stresses, suggests that MKK3 may also function as a negative regulator development in this context. It is interesting to speculate that the MKK3 signaling module may overlap with, or act in parallel to the MKK2 signaling module, which has been reported to mediate the plant response to cold and salt stress (Teige et al., 2004). Over-production of constitutively active MKK2 confers salt tolerance, while *mkk2*-null plants are hypersensitive to salt stress (Teige et al., 2004). Perhaps the MKK2 signaling module controls a more rapid response to salt and cold that dictates the immediate response of the plant, while the MKK3 signaling module may control the more subtle, long-term acclimation responses.

The available data clearly indicate that extensive cross-talk between signaling modules occurs at the MAPKK level; only MPK13 has not been shown to be phosphorylated by multiple upstream MAPKKs (Figure 5.1). However, it is also very likely that a great deal of functional characterization of these MAPK signaling modules remains to be completed since the majority of MAPKKKs and MAPKs have not been linked to any of the characterized pathways. In addition, the regulatory mechanisms that impart specificity to MAPK signaling modules must be identified in order to understand how the plant can utilize apparently overlapping MAPK signaling architectures to direct specific responses to activating stimuli.

Future directions

The reverse genetics and biochemical approaches used in my research program to identify the biological function of MKK3 signaling have revealed several apparent links between MKK3 signaling and phytohormone and stress responses. However, while it appears that MKK3 is

likely to function in the control of plant growth in response to these treatments, a precise function for MKK3 in these processes has yet to be identified. Several key experiments would further this work, both in the short- and long-term.

A) It should be a high priority to fully characterize the nature of the pair-wise interactions between MKK3 and MPK1, MPK2 and MPK7, detected in the yeast two-hybrid screens. My data indicate that MKK3 does not phosphorylate any of these putatively interacting MAPKs, but rather, it interacts with these kinases to inhibit their autoactivation. In addition, this interaction is NTF2 domain-dependent, at least in *S. cerevisiae* cells. Two experiments to verify this are:

1) verify and quantify the ability of MKK3 to inhibit the autophosphorylation activity of each of these kinases by using *in vitro* phosphorylation assays and ^{33}P-labelled ATP.
2) conduct pull-down assays of immunoprecipitated protein from *E. coli* lysates containing both recombinant MKK3 and the specific MAPK to test the ability of full-length MKK3 to interact in vitro with each of MPK1, MPK2 and MPK7.

Efforts to establish a complete AtMPK clone library are already underway so that the ability of CA-MKK3 to potentially activate those MAPKs not included in my thesis study can be examined. While several non-catalytic interactions have been identified in MAPK signaling modules in other organisms, characterization of both the substrates of MKK3 and interacting MAPKs will provide much needed information pertaining to the regulation of MAPK signaling in plants.

B) The analysis of *MKK3* gene expression using a *MKK3 promoter:GUS* reporter indicated that *MKK3* expression can be induced by auxin, by ABA, and by osmotic and heat stresses. These analyses were all completed following 24-hour treatment periods. Greater detail regarding possible functions of MKK3 in the responses to these stresses could be obtained by analysis of

MKK3 expression using a time-course of treatments followed by a recovery period. If MKK3 does function to negatively regulate growth in response to these stresses, *MKK3* expression may not be rapidly induced following exposure (i.e. *MKK3* is not an "early response" gene), but it would be assumed that increased expression would persist until sometime during the recovery period, correlated with the resumption of normal growth patterns. This would be similar to the case in yeast, where the Hog1 MAPK signaling module is rapidly activated in response to high osmolarity yet changes in gene expression of *MKK1* and *MKK2* that are involved in the cell wall remodeling MAPK module, needed for long term survival of in hyperosmotic environments, occurs at later time points (Roberts et al., 2000).

In addition to a time course evaluation of the response to auxin, ABA, salt and heat, other treatments known to inhibit growth could be added to this panel to determine how broadly MKK3 signaling functions in these processes. These experiments could ultimately be performed using real-time PCR analysis of cDNA derived from root-tips, which are the primary sites of *MKK3* expression induction. If performed by this method, the expression pattern of other MAPKs could simultaneously be examined in order to identify the MAPKs involved in these processes.

C) The gene encoding ACS6, a key ethylene biosynthetic enzyme, was down-regulated in the SALK 051970 T-DNA insertion line, as were four *ERF*s, which encode ethylene response factors. Ethylene is produced in response to environmental stresses and auxin exposure (Abel et al., 1995; Samuel et al., 2005) and the decreased expression of *ACS6* in the SALK 051970 line suggests that these plants may have impaired ethylene production in response to these stimuli. The putative link between MKK3 and ethylene could first be explored by comparing ethylene production in SALK 051970 and WT plants. If this relationship is verified, it could be further

examined using ERF4 over-expression and loss-of-function plants that display increased root growth inhibition in response to JA and ethylene, and ABA insensitivity, respectively (McGrath et al., 2005; Yang et al., 2005). *ERF4* expression was down-regulated in the SALK 051970 plants but up-regulated in the *35S:MKK3* over-expression plants, consistent with the idea that there is a functional relationship between *MKK3* and *ERF4*. It would be interesting to use the SALK 051970 plants to determine if MKK3 signaling contributes to the phenotypes observed in the ERF4 mutant lines. This would add a new layer of complexity to the previously characterized ethylene biosynthesis related MAPK modules that involve CTR1, NtMEK2 (AtMKK4/5 orthologue), AtMKK9, and AtMPK6
(Huang et al., 2003; Kim et al., 2003; Liu and Zhang, 2004; Cluis, 2005).

D) It would be very informative to identify how MKK3 signaling is regulated within the plant. It appears likely that MKK3 signaling may be controlled at least in part at a post-transcriptional level through miRNA-mediated mRNA degradation. Analysis of *MKK3* expression patterns in some of the recently reported miRNA over-expression lines (Achard et al., 2004; Guo et al., 2005; Williams et al., 2005) could clarify this. However, multiple miRNA prediction algorithms have identified at least two potential miRNAs that could specifically target *MKK3* for degradation. It would therefore be interesting to analyze *MKK3* expression patterns in transgenic plants over-expressing these putative *MKK3*-specific miRNAs to determine whether they might play a regulatory role in *MKK3* expression. If *MKK3* expression is altered in these plants, molecular and visual phenotypic analysis would also need to be conducted. It has been reported that individual miRNAs can control the expression of genes encoding proteins acting in common physiological pathways (regulons; Sunkar and Zhu, 2004; Carrington, 2005), and if this proved

to be the case for MKK3, it is possible that when multiple MKK3 signaling components are down-regulated the elusive *mkk3*-null phenotype could be revealed.

E) To help identify the hierarchical position of MKK3 signaling in response to activating stimuli, it would be useful to analyze the expression of *MKK3* in auxin-, ABA- and salt- insensitive mutants, with and without *MKK3* expression-inducing treatments.

F) Persistent *MKK3* expression in seeds that fail to develop and a failure to recover transgenic plants highly over-expressing *MKK3* under the control of the CaMV 35S promoter suggest that MKK3 signaling may significantly impair embryo and/or seed development. Prolonged developmental defects associated with the dexamethasone- inducible gene expression system precluded answering this question using the CA-MKK3 plants developed in my research program. However, to gain more insight into this phenomenon, a comparison of the transformation rates of plants with CA-MKK3, WT-MKK3 and catalytically inactive, KI-MKK3 constructs, each controlled by the CaMV 35S promoter could be completed. Catalytically inactive MAPKKs can be constructed by replacing an essential lysine residue with an arginine (Jin et al., 2003). Therefore, if active MKK3 prevents seed development, it would be expected that few, if any transgenic plants expressing the *CA-MKK3* variant, and perhaps the *WT-MKK3* would be recovered, while the recovery of several lines displaying high levels of *KI-MKK3* would be expected. However, it is possible that an overabundance of MKK3, irrespective of its activation status may impair development through a dominant negative effect. In this case, the link between *MKK3* expression and seed development would have to be examined using another inducible promoter system that allows long-term expression induction without confounding developmental defects.

G) The microarray-based transcriptional profiling experiments reported in this dissertation reflect transcriptional changes that most likely result from the absence of MKK3 in untreated SALK 051970 plants. Further microarray studies characterizing the transcriptional profile of these plants following treatment with auxin, ABA and/or salt would provide additional information concerning the involvement of MKK3 signaling in the response to these stimuli. Incorporation of several time points in these studies will also help to differentiate between early- and late-responses that involve MKK3. Because *MKK3* expression is typically induced only in the root tips, these studies should be conducted using either isolated root tips, or even better, using only cells known to show *MKK3* expression induction. These might be isolated using laser-capture microdissection, or by adopting the cell-labeling strategy used by Birnbaum et al (2003) to retrieve tissue-specific samples of cells for microarray analysis.

H) One original goal of my research program was to identify the function of the NTF2 domain encoded by MKK3. Yeast two-hybrid protein interaction studies illustrate that a function of the NTF2 domain is to mediate the pair-wise interactions between MKK3 and MPK1, MPK2 and MPK7. While the discrepancy between the requirement for the NTF2 domain to mediate these interactions in the yeast-two-hybrid and in the *in vitro* activation assays remains to be resolved, further interaction studies examining the ability of the NTF2 domain alone to interact with these proteins may provide useful insight into this phenomenon. The function of the NTF2 domain was also examined by real-time PCR- mediated transcriptional profiling experiments using transgenic plants over-expressing either full-length *MKK3* or an *MKK3ΔNTF2* variant to identify genes that appear to be specifically related to the NTF2 domain. While no clear pattern could be extracted from this short list of genes, these preliminary studies indicate that large-scale,

microarray comparisons of expression profiles between these genotypes should ultimately identify a complete set of NTF2 domain-dependent genes.

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APPENDICES

Appendix 1: General Protocols

Standard PCR

Each standard PCR reaction was set-up as follows:

Reagent	Volume/Amount
Jump Start Redi-Taq DNA Polymerase Master Mix	10 µL
Forward primer	0.5 nM
Reverse primer	0.5 nM
DNA template	10-100 ng
ddH_2O	To 20 µL

Quantitative real-time PCR

TOPO TA-mediated cloning

TOPO TA-mediated cloning reactions were set-up according to kit guidelines (Invitrogen) as follows:

Reagent	Volume/Amount
Salt mix	1 µL
TOPO TA vector	1 µL
Fresh PCR product	1 µL (~150 ng)
ddH_2O	To 6 µL

TOPO TA cloning reactions were held at room temperature for 20 minutes followed by transformation in competent *E. coli DH5Δ* cells. Positive clones were isolated first by selection on LB agar plates containing appropriate antibiotics and second by PCR analysis and DNA sequencing.

Floral dip method

A 250 mL overnight culture of *Agrobacterium tumefaciens EHA105* harbouring the binary vector containing the appropriate transgene was pelleted and resuspended in a 200 mL transformation medium (5% sucrose solution; 0.015% Silwet L-77). Flowering plants were dipped into this solution for three seconds following which plants were held in the dark in a humid environment for 24 hours. Plants were removed from this environment and returned to normal growth conditions for one week followed by re-dipping of plants. After the second dip, plants were maintained in normal growth conditions until seed set.

Appendix 2: Media Recipes and Reagent Stocks

½ MS agar plates

Reagent	Volume/Amount per litre
MS salt mixture (Sigma-Aldrich)	2.15 g
MS Vitamin mix (Sigma-Aldrich)	1 mL
Sucrose	10 g
MES buffer (Sigma-Aldrich)	0.5 g
Agar	3 g
Phytagel (Sigma-Aldrich)	1.1 g

Adjust pH to 5.6 using 1.0 N KOH
Autoclave for 20 minutes
Cool to approximately 65 C then add appropriate additives (antibiotics/hormones)

100 mM ABA Stock

Reagent	Volume/Amount
ABA (Sigma-Aldrich)	26.43 mg
Methanol	1 mL

For ½ MS agar plates with 100 μM ABA, after autoclaving 250 mL ½ MS with agar, add 250 μL of the ABA stock into media. Pour plates.

For ABA control plates repeat as above but add 250 μL methanol to the 250 mL ½ MS instead of the ABA stock.

10 mM GA Stock

Reagent	Volume/Amount
GA (Sigma-Aldrich)	3.5 mg
Ethanol	1 mL

For ½ MS agar plates with 10 μM GA, after autoclaving 250 mL ½ MS with agar, add 250 μL of the GA stock into media. Pour plates.

For GA control plates repeat as above but add 250 μL ethanol to the 250 mL ½ MS instead of the GA stock.

10 mM BR Stock

Reagent	Volume/Amount
Epi-brassinolide (Sigma-Aldrich)	2.3 mg
Glacial acetic acid	1 mL

For ½ MS agar plates with 1 μM BR, after autoclaving 250 mL ½ MS with agar, add 25 μL of the BR stock into media. Pour plates.

For BR control plates repeat as above but add 25 μL glacial acetic acid to the 250 mL ½ MS instead of the BR stock.

10 mM ACC Stock

Reagent	Volume/Amount
ACC (Sigma-Aldrich)	25.3 mg
Ethanol	1 mL

For ½ MS agar plates with 2 μM ACC, after autoclaving 250 mL ½ MS with agar, add 50 μL of the ACC stock into media. Pour plates.

For ACC control plates repeat as above but add 50 μL ethanol to the 250 mL ½ MS instead of the ACC stock.

200 mM Salicylic Acid Stock

Reagent	Volume/Amount
SA (Sigma-Aldrich)	27.6 mg
Ethanol	1 mL

For ½ MS agar plates with 200 μM SA, after autoclaving 250 mL ½ MS with agar, add 250 μL of the SA stock into media. Pour plates.

For SA control plates repeat as above but add 250 μL ethanol to the 250 mL ½ MS instead of the SA stock.

2% 1-naphthylphthalamic acid (NPA) Stock

Reagent	Volume/Amount
NPA (Sigma-Aldrich)	200 mg
DMSO	10 mL

For ½ MS agar plates with 5 μM NPA, after autoclaving 250 mL ½ MS with agar, add 91 μL of a 1/10 dilution of the NPA stock into media. Pour plates.

For NPA control plates, after autoclaving 250 mL ½ MS with agar, add 91 μL of DMSO into media. Pour plates.

10 mM Kinetin Stock

Reagent	Volume/Amount
Kinetin (Sigma-Aldrich)	21.52 mg
0.1 N NaOH	10 mL

For ½ MS agar plates with 0.5 μM kinetin, after autoclaving 250 mL ½ MS with agar, add 8 μL of the kinetin stock into media. Pour plates.

For kinetin control plates, after autoclaving 250 mL ½ MS with agar, add 8 μL of 0.1 N NaOH into media. Pour plates.

YTA Medium

Reagent	Volume/Amount per litre
Tryptone	16 g
Yeast extract	10 g
NaCl	5 g

Adjust pH to 7.0 with 1 N NaOH
Autoclave for 20 minutes
Cool to < 65 °C and add Ampicillin to a final concentration of 100 μg/mL

LB Broth

Reagent	Volume/Amount per litre
Tryptone	10 g
Yeast extract	10 g
NaCl	5 g

Adjust pH to 7.0 with 1 N NaOH
Autoclave for 20 minutes
Cool to < 65°C and add appropriate antibiotics
For LB agar plates, add 20 g Agar per litre prior to autoclaving

Appendix 3: SALK 051970/WT microarray data analysis

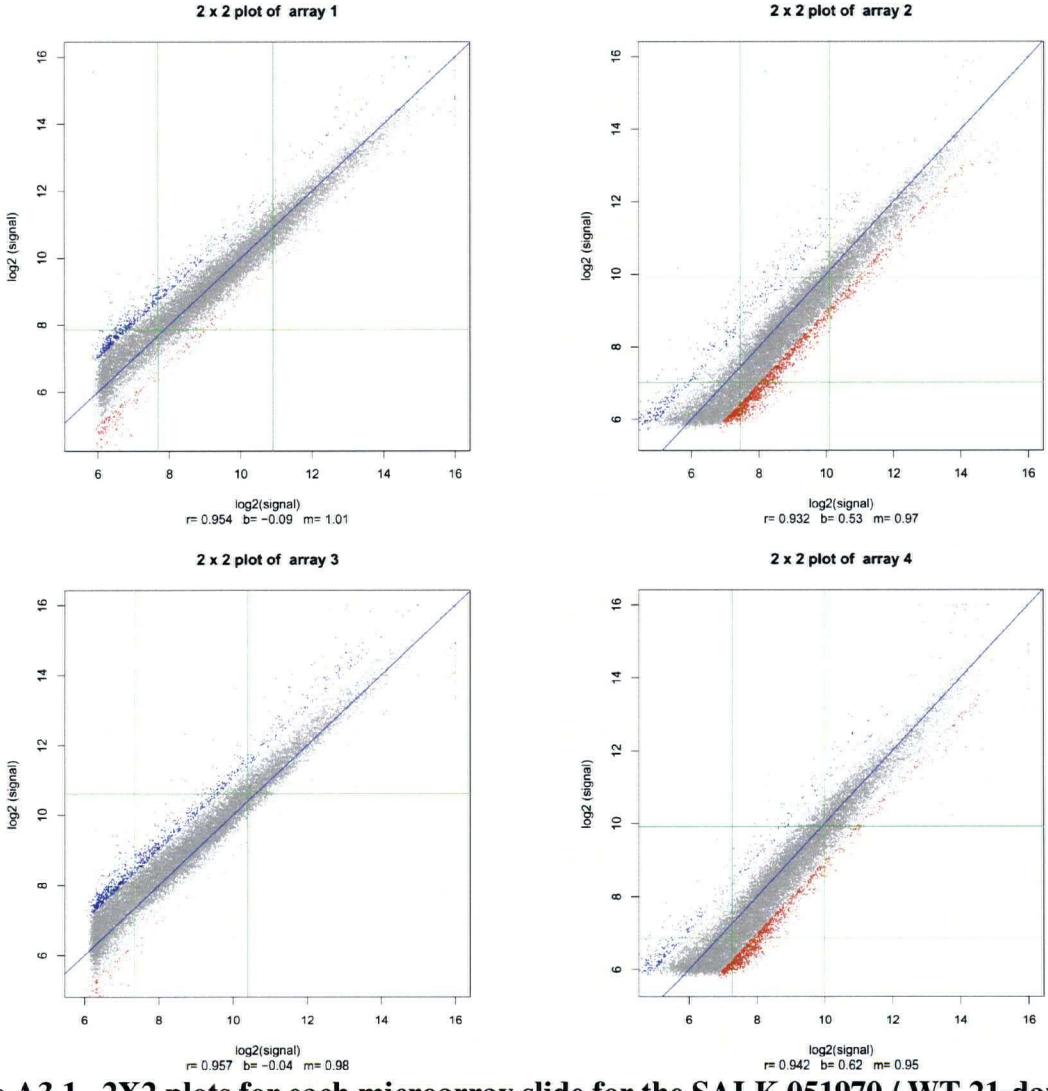

Figure A3.1. 2X2 plots for each microarray slide for the SALK 051970 / WT 21-day old pre-bolting rosette transcriptional profiling experiment. Microarrays were set-up as follows: Array 1 = SALK 051970-Cy5/WT-Cy3. Array 2 = SALK 051970-Cy3/WT-Cy5. Array 3 = SALK 051970-Cy5/WT-Cy3. Array 4 = SALK 051970-Cy3/WT-Cy5.

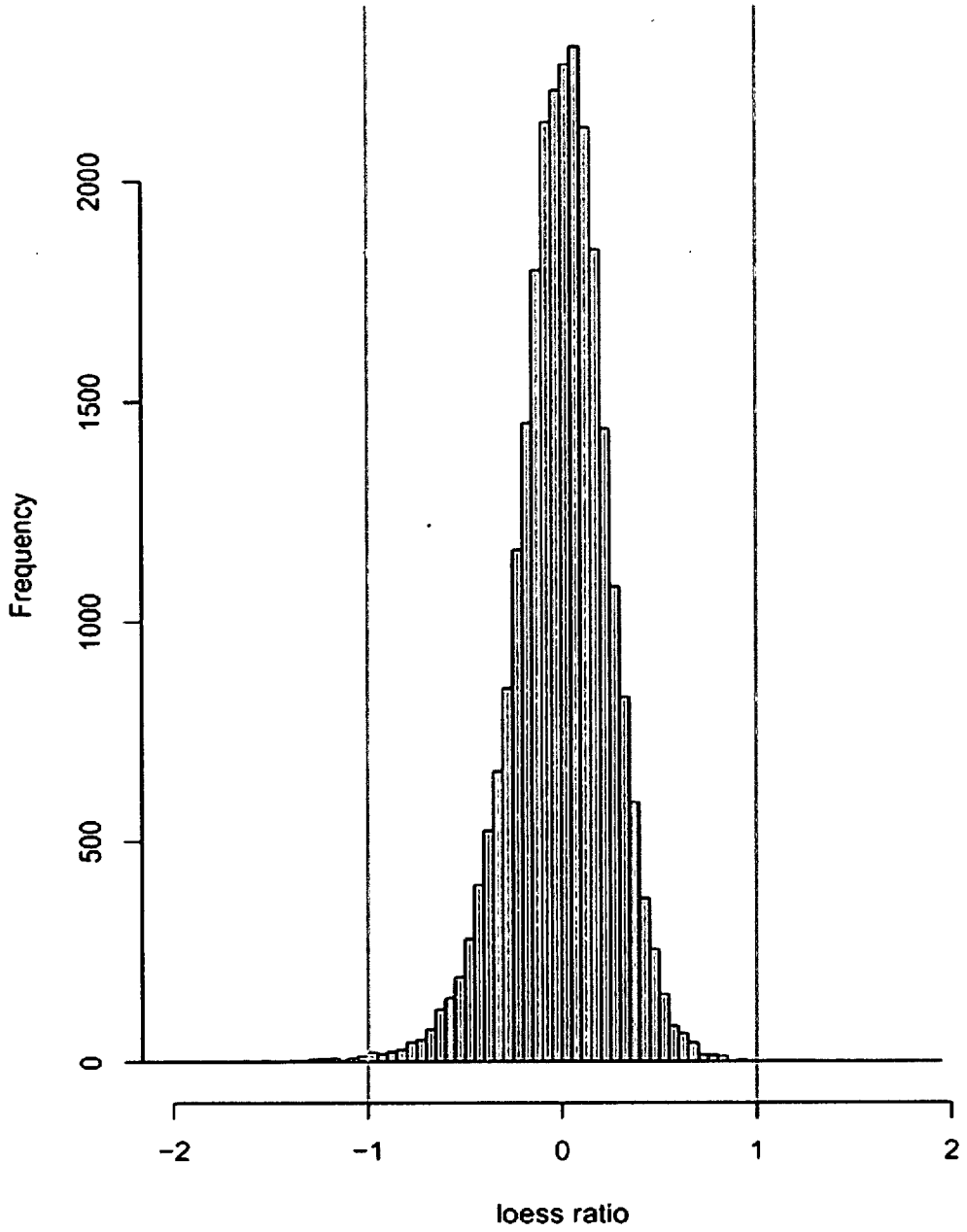

Figure A3.2: Distribution of loess ratios for the SALK 051970 / WT 21-day old pre-bolting rosette transcriptional profiling experiment. The ratios show a largely normal distribution, with very few points showing loess ratios greater than +/- 1.0, a common two-fold expression differential cutoff used in many microarray experiments. In order to extract more data from the experiment, the fold-change cutoff was expanded to +/- 1.75X for subsequent data analysis.

Appendix 4. PCR primers used in the real-time PCR study

Sequences of PCR primers used to determine the expression profiles of MKK3-associated genes in the SALK 051970 and MKK3-variant over-expression lines using real-time PCR. All primers are complementary to sequences in successive exons to enable detection of contaminating genomic DNA in cDNA samples.

Locus	Forward Primer	Reverse Primer
At3g48360	CGCAGTTTAAGACCGTTGAG	GATGGACCGACCAATGTAC
At4g27410	AACATTCTCGTAGCCATGG	AAGAACGTCGTCAAGCTGTG
At1g80840	TGGACAGAAAGTGACTAGAG	TGAAGCTGAACCACCATGAG
At1g69490	GCATGAGTATCGTCTCCATG	TCCTCCATTAGTACTTCGTC
At2g38470	CATCGATTGTCAGCAGAGAC	TGTGATGCTCTCTCCACATG
At4g39060	AGCTGACTGGCAACGGTC	CTTCTTCTTGATCGTCACAC
At1g27730	AGCTCTCGGCGGACACAAG	GGCATAGGGCTCATGACTTC
At2g46680	AGCTCGAGACTGAGTACAAC	TCTCCACTACACTGCCTTTC
At1g53170	AGTCCTACTCAGAGTAGCAC	TCTTTGGATACGTCGCCATG
At4g23190	AGCGGCGTCTACCAGATG	CAGCGCGATATGGATGC
At1g28370	TGAGTTTGACACAGAGCCTG	TCACCACCGACGAAGAATCC
At3g11410	ATTCGGATCCAACAAGCTGG	CCAAGATCAAACACTCATCC
At3g52400	CGATCACGAATGAGTAC	TCTTTCACTGCGTCGTG
At5g04340	GCCACCGGTCAAGCTCT	GGCATAGGACTCATCACCT
At3g15210	TCCTCCGACGTTAGTTGT	GACGAATCAGAGTCGCT
At4g31550	TGCTTCTGCTCCGTTGCA	ACTTATCGCCGGTACTCT
At3g19680	GTGAGCCTGAGTGTAGTCC	GCTTATCTCAGACATCATCG
At1g14210	GAGTGGAATAAGCATGGCA	GTGCAAATCCGATCGCGCA
At2g21650	CATCGAGAATGGTCACGTG	CTGCAGCTTCATGCTTCT
At4g38840	CTTCTTCTAGCTCTCTTGA	GAAGCGAGAAGCAAGATCA
At5g42040	ACCTGATGAGACTTACGT	TGTTGATCAGACGAGAAGA
At2g07020	GAATCTGAGATGAGAAGGCT	CATTGCTGCTTCTTTGGCA
Actin 1	CGATGAAGCTCAATCCAAACGA	CAGAGTCGAGCACAATACCG

Appendix 5: CAREs in the MKK3 promoter sequences

CARE Name	Description	Location	Sequence
ABRELATERD1	Responsive to dehydration	-551	ACGTG
ACGTATERD1	Responsive to dehydration	-1228	ACGT
ACGTATERD1	Responsive to dehydration	-1018	ACGT
ACGTATERD1	Responsive to dehydration	-883	ACGT
ACGTATERD1	Responsive to dehydration	-861	ACGT
ACGTATERD1	Responsive to dehydration	-550	ACGT
ACGTATERD1	Responsive to dehydration	-1228	ACGT
ACGTATERD1	Responsive to dehydration	-1018	ACGT
ACGTATERD1	Responsive to dehydration	-883	ACGT
ACGTATERD1	Responsive to dehydration	-861	ACGT
ACGTATERD1	Responsive to dehydration	-550	ACGT
ARFAT	ARF binding site	-755	TGTCTC
ARFAT	ARF binding site	-1043	TGTCTC
ARFAT	ARF binding site	-447	TGTCTC
ARR1AT	ARR binding site	-265	NGATT
ARR1AT	ARR binding site	-1430	NGATT
ARR1AT	ARR binding site	-1332	NGATT
ARR1AT	ARR binding site	-808	NGATT
ARR1AT	ARR binding site	-318	NGATT
ARR1AT	ARR binding site	-287	NGATT
ARR1AT	ARR binding site	-1363	NGATT
ARR1AT	ARR binding site	-857	NGATT
ARR1AT	ARR binding site	-827	NGATT
ARR1AT	ARR binding site	-217	NGATT
ARR1AT	ARR binding site	-204	NGATT
ARR1AT	ARR binding site	-111	NGATT
CATATGGMSAUR	Found in SAUR promoters	-993	CATATG
CATATGGMSAUR	Found in SAUR promoters	-993	CATATG
CCAATBOX1	Found in heat shock promoters	-1257	CCAAT
CCAATBOX1	Found in heat shock promoters	-201	CCAAT
CCAATBOX1	Found in heat shock promoters	-871	CCAAT
CCAATBOX1	Found in heat shock promoters	-741	CCAAT
CCAATBOX1	Found in heat shock promoters	-631	CCAAT
CELLCYCLESC	Cell cycle related	-624	CACGAAAA
CIACADIANLELHC	Circadian expression	-807	CAANNNNATC
CIACADIANLELHC	Circadian expression	-317	CAANNNNATC
DPBFCOREDCDC3	ABA inducible/embryo specific	-1168	ACACNNG
DPBFCOREDCDC3	ABA inducible/embryo specific	-521	ACACNNG
EECCRCAH1	Myb binding site	-807	GANTTNC
EECCRCAH1	Myb binding site	-770	GANTTNC
EECCRCAH1	Myb binding site	-504	GANTTNC
EECCRCAH1	Myb binding site	-1099	GANTTNC
EECCRCAH1	Myb binding site	-352	GANTTNC

CARE Name	Description	Location	Sequence
ERELEE4	Ethylene responsive element	-311	AWTTCAAA
GAREAT	GA responsive	-374	TAACAAR
GAREAT	GA responsive	-125	TAACAAR
IBOXCORE	Light regulated	-358	GATAA
IBOXCORE	Light regulated	-4	GATAA
LTRE1HVBLT49	Low temperature inducible	-1001	CCGAAA
LTRECOREATCOR15	Low temperature inducible	-508	CCGAC
MYB1AT	Myb binding site	-1151	WAACCA
MYB1AT	Myb binding site	-792	WAACCA
MYB1AT	Myb binding site	-430	WAACCA
MYB1AT	Myb binding site	-1116	WAACCA
MYB1AT	Myb binding site	-925	WAACCA
MYB1AT	Myb binding site	-328	WAACCA
MYB2AT	Myb binding site	-234	TAACTG
MYB2CONSENSUSAT	Myb binding site	-234	YAACKG
MYB2CONSENSUSAT	Myb binding site	-291	YAACKG
MYBCORE	Myb binding site	-291	CNGTTR
MYBCORE	Myb binding site	-254	CNGTTR
MYBCORE	Myb binding site	-234	CNGTTR
MYBGAHV	Myb binding site	-374	TAACAAA
MYBGAHV	Myb binding site	-125	TAACAAA
MYBPLANT	Myb binding site	-429	MACCWAMC
MYBPLANT	Myb binding site	-1155	MACCWAMC
MYBPZM	Myb binding site	-51	CCWACC
MYCCONSENSUSAT	Myc binding site/dehydration	-993	CANNTG
MYCCONSENSUSAT	Myc binding site/dehydration	-812	CANNTG
MYCCONSENSUSAT	Myc binding site/dehydration	-750	CANNTG
MYCCONSENSUSAT	Myc binding site/dehydration	-719	CANNTG
MYCCONSENSUSAT	Myc binding site/dehydration	-993	CANNTG
MYCCONSENSUSAT	Myc binding site/dehydration	-812	CANNTG
MYCCONSENSUSAT	Myc binding site/dehydration	-750	CANNTG
MYCCONSENSUSAT	Myc binding site/dehydration	-719	CANNTG
NTBBF1ARROLB	Auxin inducible	-1481	ACTTTA
NTBBF1ARROLB	Auxin inducible	-906	ACTTTA
POLLEN1LELAT52	Pollen specific	-689	AGAAA
POLLEN1LELAT52	Pollen specific	-487	AGAAA
POLLEN1LELAT52	Pollen specific	-434	AGAAA
POLLEN1LELAT52	Pollen specific	-179	AGAAA
POLLEN1LELAT52	Pollen specific	-157	AGAAA
POLLEN1LELAT52	Pollen specific	-114	AGAAA
POLLEN1LELAT52	Pollen specific	-75	AGAAA
POLLEN1LELAT52	Pollen specific	-58	AGAAA
POLLEN1LELAT52	Pollen specific	-15	AGAAA
POLLEN1LELAT52	Pollen specific	-1005	AGAAA
POLLEN1LELAT52	Pollen specific	-778	AGAAA
POLLEN1LELAT52	Pollen specific	-271	AGAAA
POLLEN1LELAT52	Pollen specific	-87	AGAAA
PREATPRODH	Hypoosmolarity responsive	-1476	ACTCAT
PREATPRODH	Hypoosmolarity responsive	-1053	ACTCAT

CARE Name	Description	Location	Sequence
TBOXATGAPB	Light regulated	-384	ACTTTG
TBOXATGAPB	Light regulated	-210	ACTTTG
WBBOXPCWRKY1	WRKY binding site	-1107	TTTGACT
WBOXATNPR1	WRKY binding site	-1491	TTGAC
WBOXATNPR1	WRKY binding site	-1486	TTGAC
WBOXATNPR1	WRKY binding site	-1106	TTGAC
WBOXATNPR1	WRKY binding site	-223	TTGAC
WBOXATNPR1	WRKY binding site	-35	TTGAC
WBOXNTERF3	WRKY binding site	-1490	TGACY
WBOXNTERF3	WRKY binding site	-1105	TGACY
WBOXNTERF3	WRKY binding site	-943	TGACY
WBOXNTERF3	WRKY binding site	-695	TGACY
WBOXNTERF3	WRKY binding site	-222	TGACY
WBOXNTERF3	WRKY binding site	-34	TGACY

Appendix 6. Multiple sequence alignment of AtMPKs

AtMPK sequences were aligned using ClustalW Version 1.83. TEY and TDY motifs are highlighted (yellow and red), as are putative MKK3 interaction domains in MPK1, MPK2 and MPK7 (green, cyan). Corresponding domains in the TDY-containing MAPK sequences are highlighted in blue.

```
CLUSTAL W (1.83) multiple sequence alignment


AtMPK7          ----------------------------------------MAMLVEPP---------- 8
AtMPK14         ----------------------------------------MAMLVDPP---------- 8
AtMPK1          ----------------------------------------MATLVDPP---------- 8
AtMPK2          ----------------------------------------MATPVDPP---------- 8
AtMPK4          ----------------------------------------MSAESCFGS--------- 9
AtMPK11         ----------------------------------------MSIEKPF---------- 7
AtMPK12         ----------------------------------------MSGESSS---------- 7
AtMPK5          ----------------------------------------MAKEIESAT--------- 9
AtMPK13         ----------------------------------------MEKREDG---------- 7
AtMPK3          ----------------------------------------MNTGGGQ---------- 7
AtMPK6          ----------------------------------------MDGGSGQPAADTEMTEAP 18
AtMPK10         ----------------------------------------MEPTNDAETLETQGEVTT 18
AtMPK8          MGGGGNLVDGVRRWLFQRPSSSSSSSSSNNNNNNHEQPIFNSSSFSSSSNPNHSANSGEL 60
AtMPK15         MGGGGNLVDGVRRWLFFQRRPSSSSSS-----NNHDQ-IQNPPTVS---NPNDDED---- 47
AtMPK17         ------------------------------------------------------------
AtMPK16         ------------------------------------------------------------
AtMPK9          ------------------------------------------------------------
AtMPK18         ------------------------------------------------------------
AtMPK19         ------------------------------------------------------------
AtMPK20         ------------------------------------------------------------


AtMPK7          ------------------NGIKQQGKHY--YSMWQTLFEIDTKYVP-IKPIGRGAYGVVC 47
AtMPK14         ------------------NGIRQEGKHY--YTMWQTLFEIDTKYVP-IKPIGRGAYGVVC 47
AtMPK1          ------------------NGIRNEGKHY--FSMWQTLFEIDTKYMP-IKPIGRGAYGVVC 47
AtMPK2          ------------------NGIRNQGKHY--FSMWQTLFEIDTKYMP-IKPIGRGAYGVVC 47
AtMPK4          -----------SGDQSSSKGVATHGGSYVQYNVYGNLFEVSRKYVPPLRPIGRGAYGIVC 58
AtMPK11         -----------FGDDSN-RGVSINGGRYVQYNVYGNLFEVSKKYVPPLRPIGRGASGIVC 55
AtMPK12         -----------GSTEHCIKVVPTHGGRYVQYNVYGQLFEVSRKYVPPIRPIGRGACGIVC 56
AtMPK5          -----------DLGDTNIKGVLVHGGRYFQYNVYGNLFEVSNKYVPPIRPIGRGAYGFVC 58
AtMPK13         ------------------GILTYDGRYVMYNVLGNIFELSSKYIPPIEPIGRGAYGIVC 48
AtMPK3          --------------YTDFPAVETHGGQFISYDIFGSLFEITSKYRPPIIPIGRGAYGIVC 53
AtMPK6          GGFPAAAPSPQMPGIENIPATLSHGGRFIQYNIFGNIFEVTAKYKPPIMPIGKGAYGIVC 78
AtMPK10         AIWPS---SQILKTTIDIPGTLSHDGRYIQYNLFGHIFELPAKYKPPIRPIGRGACGIVC 75
AtMPK8          IIEEDLDFSGLTLINVPKRNHLPMDPHKKGETEFFTEYGEANRYQI-QEVVGKGSYGVVA 119
AtMPK15         -LKKLTDPSKLRQIKVQQRNHLPMEKKGIPNAEFFTEYGEANRYQI-QEVVGKGSYGVVG 105
AtMPK17         ---------------------------MLEKEFFTEYGEASQYQI-QEVVGKGSYGVVA 31
AtMPK16         ----------------MQPDHR---KKSSVEVDFFTEYGEGSRYRI-EEVIGKGSYGVVC 40
AtMPK9          -------------------------------MEFFTEYGDANRYRI-LEVIGKGSYGVVC 28
AtMPK18         ------------------MQQNQVKKGTKEMEFFTEYGDANRYRI-LEVIGKGSYGVVC 40
AtMPK19         -------------------------------MEFFTEYGDANRYRI-LEVIGKGSYGVVC 28
AtMPK20         ------------------MQQDNRKKNNLEMEFFSDYGDANRFKV-QEVIGKGSYGVVC 40
                                                :    ::      :*:*: *.*
```


AtMPK7 SSINRETNERVAIKKIHNVFENRVDALRTLRELKLLRHVRHENVIALKDVMLPANRSSFK 107
AtMPK14 SSINSETNERVAIKKIHNVFENRIDALRTLRELKLLRHVRHENVISLKDVMLPTHRYSFR 107
AtMPK1 SSVNSDTNEKVAIKKIHNVYENRIDALRTLRELKLLRHLRHENVIALKDVMMPIHKMSFK 107
AtMPK2 SSVNRESNERVAIKKIHNVFENRIDALRTLRELKLLRHLRHENVVALKDVMMANHKRSFK 107
AtMPK4 AATNSETGEEVAIKKIGNAFDNIIDAKRTLREIKLLKHMDHENVIAVKDIIKPPQRENFN 118
AtMPK11 AAWNSETGEEVAIKKIGNAFGNIIDAKRTLREIKLLKHMDHDNVIAIIDIIRPPQPDNFN 115
AtMPK12 AAVNSVTGEKVAIKKIGNAFDNIIDAKRTLREIKLLRHMDHENVITIKDIVRPPQRDIFN 116
AtMPK5 AAVDSETHEEIAIKKIGKAFDNKVDAKRTLREIKLLRHLEHENVVVIKDIIRPPKKEDFV 118
AtMPK13 CATNSETNEEVAIKKIANAFDNRVDAKRTLREIKLLSHMDHDNVIKIKDIIELPEKERFE 108
AtMPK3 SVLDTETNELVAMKKIANAFDNHMDAKRTLREIKLLRHLDHENIIAIRDVVPPPLRRQFS 113
AtMPK6 SAMNSETNESVAIKKIANAFDNKIDAKRTLREIKLLRHMDHENIVAIRDIIPPPLRNAFN 138
AtMPK10 SAVDSETNEKVAIKKITQVFDNTIEAKRTLREIKLLRHFDHENIVAIRDVILPPQRDSFE 135
AtMPK8 SAVDSHTGERVAIKKINDVFEHVSDATRILREIKLLRLLRHPDVVEIKHIMLPPSRREFR 179
AtMPK15 SAIDTHTGERVAIKKINDVFDHISDATRILREIKLLRLLLHPDVVEIKHIMLPPSRREFR 165
AtMPK17 SAECPHTGGKVAIKKMTNVFEHVSDAIRILREIKLLRLLRHPDIVEIKHIMLPPCRKEFK 91
AtMPK16 SAYDTHTGEKVAIKKINDIFEHVSDATRILREIKLLRLLRHPDIVEIKHILLPPSRREFR 100
AtMPK9 AAIDTHTGEKVAIKKINDVFEHISDALRILREVKLLRLLRHPDIVEIKSIMLPPSKREFK 88
AtMPK18 AAIDTHTGEKVAIKKINDVFEHISDALRILREVKLLRLLRHPDIVEIKSIMLPPSKREFK 100
AtMPK19 AAIDTQTGEKVAIKKINDVFEHVSDALRILREVKLLRLLRHPDIVEIKSIMLPPSKREFK 88
AtMPK20 SAIDTLTGEKVAIKKIHDIFEHISDAARILREIKLLRLLRHPDIVEIKHIMLPPSRREFK 100
AtMPK7 DVYLVYELMDTDLHQIIKSSQSLSDDHCKYFLFQLLRGLKYLHSANILHRDLKPGNLLVN 167
AtMPK14 DVYLVYELMDSDLNQIIKSSQSLSDDHCKYFLFQLLRGLKYLHSANILHRDLKPGNLLVN 167
AtMPK1 DVYLVYELMDTDLHQIIKSSQVLSNDHCQYFLFQLLRGLKYIHSANILHRDLKPGNLLVN 167
AtMPK2 DVYLVYELMDTDLHQIIKSSQVLSNDHCQYFLFQLLRGLKYIHSANILHRDLKPGNLLVN 167
AtMPK4 DVYIVYELMDTDLHQIIRSNQPLTDDHCRFFLYQLLRGLKYVHSANVLHRDLKPSNLLLN 178
AtMPK11 DVHIVYELMDTDLHHIIRSNQPLTDDHSRFFLYQLLRGLKYVHSANVLHRDLKPSNLLLN 175
AtMPK12 DVYIVYELMDTDLQRILRSNQTLTSDQCRFLVYQLLRGLKYVHSANILHRDLRPSNVLLN 176
AtMPK5 DVYIVFELMDTDLHQIIRSNQSLNDDHCQYFLYQILRGLKYIHSANVLHRDLKPSNLLLN 178
AtMPK13 DVYIVYELMDTDLHQIIRSTQTLTDDHCQYFLYQILRGLKYIHSANVLHRDLKPSNLVLN 168
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AtMPK6 DVYIAYELMDTDLHQIIRSNQALSEEHCQYFLYQILRGLKYIHSANVLHRDLKPSNLLLN 198
AtMPK10 DVYIVNELMEFDLYRTLKSDQELTKDHGMYFMYQILRGLKYIHSANVLHRDLKPSNLLLS 195
AtMPK8 DIYVVFELMESDLHQVIKANDDLTPEHYQFFLYQLLRGLKYVHAANVFHRDLKPKNILAN 239
AtMPK15 DVYVVFELMESDLHQVIKANDDLTPEHHQFFLYQLLRGLKYVHAANVFHRDLKPKNILAN 225
AtMPK17 DIYVVFELMESDLHHVLKVNDDLTPQHHQFFLYQLLRGLKFMHSAHVFHRDLKPKNILAN 151
AtMPK16 DIYVVFELMESDLHQVIKANDDLTPEHYQFFLYQLLRGLKYIHTANVFHRDLKPKNILAN 160
AtMPK9 DIYVVFELMESDLHQVIKANDDLTREHHQFFLYQMLRALKFMHTANVYHRDLKPKNILAN 148
AtMPK18 DIYVVFELMESDLHQVIKANDDLTREHHQFFLYQMLRALKFMHTANVYHRDLKPKNILAN 160
AtMPK19 DIYVVFELMESDLHQVIKANDDLTREHHQFFLYQMLRALKYMHTANVYHRDLKPKNILAN 148
AtMPK20 DIYVVFELMESDLHQVIKANDDLTREHYQFFLYQLLRALKYIHTANVYHRDLKPKNILAN 160
AtMPK7 ANCDLKICDFGLARTSQG---NEQFMTEYVVTRWYRAPELLLC-CDNYGTSIDVWSVGCI 223
AtMPK14 ANCDLKICDFGLART------YEQFMTEYVVTRWYRAPELLLC-CDNYGTSIDVWSVGCI 220
AtMPK1 ANCDLKICDFGLARASNT---KGQFMTEYVVTRWYRAPELLLC-CDNYGTSIDVWSVGCI 223
AtMPK2 ANCDLKICDFGLARTSNT---KGQFMTEYVVTRWYRAPELLLC-CDNYGTSIDVWSVGCI 223
AtMPK4 ANCDLKLGDFGLARTKS----ETDFMTEYVVTRWYRAPELLLN-CSEYTAAIDIWSVGCI 233
AtMPK11 ANCDLKIGDFGLARTKS----ETDFMTEYVVTRWYRAPELLLN-CSEYTAAIDIWSVGCI 230
AtMPK12 SKNELKIGDFGLARTTS----DTDFMTEYVVTRWYRAPELLLN-CSEYTAAIDIWSVGCI 231
AtMPK5 SNCDLKITDFGLARTTS----ETEYMTEYVVTRWYRAPELLLN-SSEYTSAIDVWSVGCI 233
AtMPK13 TNCDLKICDFGLARTSN----ETEIMTEYVVTRWYRAPELLLN-SSEYTGAIDIWSVGCI 223
AtMPK3 ANCDLKICDFGLARPTS----ENDFMTEYVVTRWYRAPELLLN-SSDYTAAIDVWSVGCI 228
AtMPK6 ANCDLKICDFGLARVTS----ESDFMTEYVVTRWYRAPELLLN-SSDYTAAIDVWSVGCI 253
AtMPK10 TQCDLKICDFGLARATP----ESNLMTEYVVTRWYRAPELLLG-SSDYTAAIDVWSVGCI 250
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AtMPK15 ADCKLKICDFGLARVSFNDAPTAIFWTDYVATRWYRAPELCGSFFSKYTPAIDIWSVGCI 285
AtMPK17 ADCKIKICDLGLARVSFTDSPSAVFWTDYVATRWYRAPELCGSFYSNYTPAIDMWSVGCI 211
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AtMPK18 ANCKLKVCDFGLARVAFNDTPTTVFWTDYVATRWYRAPELCGSFFSKYTPAIDVWSIGCI 220
AtMPK19 ANCKLKVCDFGLARVSFNDTPTTVFWTDYVATRWYRAPELCGSFCSKYTPAIDIWSIGCI 208
AtMPK20 ANCKLKICDFGLARVAFNDTPTTIFWTDYVATRWYRAPELCGSFYSKYTPAIDIWSIGCI 220

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AtMPK14         FAEILGRKPIFPGTECLNQLKLIINVVGSQQDWDLQFIDNQKARRFIKSLPFSKGTHFSH 280
AtMPK1          FAELLGRKPIFQGTECLNQLKLIVNILGSQREEDLEFIDNPKAKRYIRSLPYSPGMSLSR 283
AtMPK2          FAELLGRKPVFPGTECLNQIKLIINILGSQREEDLEFIDNPKAKRYIESLPYSPGISFSR 283
AtMPK4          LGETMTREPLFPGKDYVHQLRLITELIGSPDDSSLGFLRSDNARRYVRQLPQYPRQNFAA 293
AtMPK11         LGEIMTREPLFPGRDYVQQLRLITEVN------------------------------- 257
AtMPK12         LGEIMTGQPLFPGKDYVHQLRLITEVYQ------------------------------ 259
AtMPK5          FAEIMTREPLFPGKDYVHQLKLITELIGSPDGASLEFLRSANARKYVKELPKFPRQNFSA 293
AtMPK13         FMEILRRETLFPGKDYVQQLKLITEVSKLKP---------------------------- 254
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AtMPK6          FMELMDRKPLFPGRDHVHQLRLLMELIGTPSEEELEFLN-ENAKRYIRQLPPYPRQSITD 312
AtMPK10         FMEIMNREPLFPGKDQVNQLRLLLELIGTPSEEELGSLS-EYAKRYIRQLPTLPRQSFTE 309
AtMPK8          FAEMLLGKPLFPGKNVVHQLDLMTDFLGTPPPESISRIRNEKARRYLSSMRKKQPVPFSH 359
AtMPK15         FAEMLLGKPLFPGKNVVHQLDIMTDFLGTPPPEAISKIRNDKARRYLGNMRKKQPVPFSK 345
AtMPK17         FAEMLTGKPLFPGKNVVHQLELVTDLLGTPSPITLSRIRNEKARKYLGNMRRKDPVPFTH 271
AtMPK16         FAELLTGKPLFPGKNVVHQLDLMTDMLGTPSAEAIGRVRNEKARRYLSSMRKKKPIPFSH 280
AtMPK9          FAEVLTGKPLFPGKSVVHQLELITDLLGTPKSETISGVRNDKARKYLTEMRKKNPVTFSQ 268
AtMPK18         FAEVLTGKPLFPGKSVVHQLELITDLLGTPKSETISGVRNDKARKYLTEMRKKNPVTFSQ 280
AtMPK19         FAEVLTGKPLFPGKSVVHQLDLITDLLGTPKSETIAGVRNEKARKYLNEMRKKNLVPFSQ 268
AtMPK20         FAEVLMGKPLFPGKNVVHQLDLMTDLLGTPSLDTISRVRNEKARRYLTSMRKKPPIPFAQ 280
                : * :  :.:* * . ::*: :: :.

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AtMPK14         IYPHANPLAIDLLQRMLVFDPTKRISVSDALLHPYMEGLLEPECNPSENVPVSSLEIDEN 340
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AtMPK2          LYPGANVLAIDLLQKILVLDPSKRISVTEALQHPYMAPLYDPSANPPAQVPID-LDVDED 342
AtMPK4          RFPNMSAGAVDLLEKMLVFDPSRRITVDEALCHPYLAPLHDINEEPVCVRPFN-FDFEQP 352
AtMPK11         ---------FSLFHLTILFR-------------------------------FN-LKKEH- 275
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AtMPK10         KFPNVPPLAIDLVEKMLTFDPKQRISVKEALAHPYLSSFHDITDEPECSEPFN-FDLDEH 368
AtMPK8          KFPKADPLALRLLERLLAFDPKDRASAEDALADPYFSGLSNSEREPTTQ-PISKLHFDFE 418
AtMPK15         KFPKADPSALRLLERLIAFDPKDRPSAEEALADPYFNGLSSKVREPSTQ-PISKLHFEFE 404
AtMPK17         KFPNIDPVALKLLQRLIAFDPKDRPSAEEALADPYFQGLANVDYEPSRQ-PISKLHFEFE 330
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AtMPK18         KFSKADPLALRLLQRLLAFDPKDRPTPAEALADPYFKGLSKIEREPSSQ-QISKMHFEFE 339
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AtMPK14         ---MEGDMIREMMWEEMLHYLPRA---------------------------------- 361
AtMPK1          ---LREEMIREMMWNEMLHYHPQASTLN----TEL----------------------- 370
AtMPK2          ED-LGAEMIRELMWKEMIHYHPEAATINNNEVSEF----------------------- 376
AtMPK4          T--LTEENIKELIYRETVKFNPQDSV--------------------------------- 376
AtMPK11         ------------------------------------------------------------
AtMPK12         ------------------------------------------------------------
AtMPK5          S--STEEEIKELVWLESVKFNPLPSI--------------------------------- 376
AtMPK13         ------------------------------------------------------------
AtMPK3          P--LDEEQIKEMIYQEAIALNPTYG---------------------------------- 370
AtMPK6          A--LSEEQMKELIYREALAFNPEYQQ--------------------------------- 395
AtMPK10         P--FSEEQFRELIYCEALAFNPETSND-------------------------------- 393
AtMPK8          RKKLVKDDVRELIYREILEYHPQMLEEYLRGGDQL--SFMYPSGVDRFKRQFAHLEENQG 476
AtMPK15         RKKLTKDDIRELIYREVMS-------------------LLYISSLV------------- 431
AtMPK17         RRKLTRDDVRELMYREILEYHPQMLQEYLQGEENINSHFLYPSGVDQFKQEFARLEEHND 390
AtMPK16         RRRITKEDVRELIYRESLEYHPKMLKEYLDGSEPTN--FMYPSAVEHFKKQFAYLEEHYK 397
AtMPK9          RRRLTKDDIRELIYREILEYHPQLLKDYMSGSEGSN--FVYPSAIGHLRQQFTYLEENSS 385
AtMPK18         RRRLTKDDIRELIYREILEYHPQLLKDYMSGSEGSN--FVYPSAIGHLRQQFTYLEENSS 397
AtMPK19         RRRLTKDDIRELIYREILEYHPQLLKDYMN-SEGSS--FLYPSAIGHLRKQFAYLEENSG 384
AtMPK20         RRKVTKEDIRELISREILEYHPQLLKDHMNGADKAS--FLYPSAVDQFRRQFAHLEENSG 397
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AtMPK10         ------------------------------------------------------------
AtMPK8          KPGAAGGGRSTALHRHHASLPRERVPAPNG------ETAEESSDVERRAAA--------- 521
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AtMPK17         DEEEHN---SPPHQRKYTSLPRERVCSSED------EGSDSVHAQSSSASV--------- 432
AtMPK16         NGTSHN----PPERQQHASLPRACVLYSDNNHPVAQQSSAEVTDGLSKCSIRDERPRGAD 453
AtMPK9          RNGPVI-----PLERKHASLPRS-TVHSTVVHS--TSQPNLGATDSRRVSFEPSKNGASS 437
AtMPK18         RNGPVI-----PLERKHASLPRS-TVHSTVVHS--TSQPNLGATDSRRVSFEPSKNGASS 449
AtMPK19         KSGPVI-----PPDRKHASLPRS-AVHSSAVNS--NAQPSLNASDSRRVSIEPSRNGVV- 435
AtMPK20         KTGPVA-----PLERKHASLPRSTVIHSTAVAR--GGQPKLMNNTN---TLNPET----- 442

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AtMPK17         ----------------------------VFTPPQTPNTATGLSSQK--------------- 450
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AtMPK18         AGHPSTSAYPTKSIGPPPRVPPSGRPGRVVESSVSYENGRNLKEA-------YFRSAVS- 501
AtMPK19         ---PSTSAYSTKPLGPPPRVP-SGKPGRVVESSVTYENDRNLKESSYDARTSYYRSTVLP 491
AtMPK20         ---TQNIPFNHATIQAQQRNLSAAKPSTFMGPVAPFDNGRISRDAYD------PRSFIR- 492

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AtMPK17         ----ASQVDK--------AATPVKRSACLMRS-----DSICAS----------------- 476
AtMPK16         ----ALEQQQRRMVRNPAAASQYPKRTQPCKSNRGDEDCATAA----------------- 538
AtMPK9          ----SPHCYFRPNTMTNPENRNIEASSFPPKPQNPVHQFSPTEPPAATTNQADVETMNHP 545
AtMPK18         ----SPHCYFRPNTMTNPENRNIEASSFPPKPQNPVHQFSPTEPPAATTNQADVETMNHP 557
AtMPK19         PQTVSPNCYFLPNTMNQEKRSGTEAASQP-KPQ-----FVPTQ-----CNSAKPAELN-P 539
AtMPK20         ----STNLPFSQQSAATVAMGKQQERRTTMEPEKQARQISQYN-----RYAPDVAINIDN 543
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AtMPK7          ------------------------------------------------------------
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AtMPK1          ------------------------------------------------------------
AtMPK2          ------------------------------------------------------------
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AtMPK6          ------------------------------------------------------------
AtMPK10         ------------------------------------------------------------
AtMPK8          ----------------------EDNETVAELTDKVASLHNS---------------------- 589
AtMPK15         ------------------------------------------------------------
AtMPK17         ----------------------RCVGVSSAVS---------------------------- 486
AtMPK16         ----------------------EGPSRLKPNTQYIPQKVSAAQDTAMSRWY---------- 567
AtMPK9          NPYFQPQLPKTDQLNNNTHMAIDAKLLQAQSQFGPAGAAAVAV---AAHRNIGTISYSAA 602
AtMPK18         NPYFQPQLPKTDQLNNNTHMAIDAKLLQAQSQFGPAGAAAVAV---AAHRNIGTISYSAA 614
AtMPK19         NPYVQSQH----------KVGIDAKLLHAQSQYGPAGAAAVAV---AAHRNIGAVGYGMS 586
AtMPK20         NPFIMARTGMNKAENISDRIIIDTNLLQATAGIGVAAAAAAAAPGGSAHRKVGAVRYGMS 603

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AtMPK1          ---
AtMPK2          ---
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AtMPK12         ---
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AtMPK3          ---
AtMPK6          ---
AtMPK10         ---
AtMPK8          ---
AtMPK15         ---
AtMPK17         ---
AtMPK16         ---
AtMPK9          S-- 603
AtMPK18         S-- 615
AtMPK19         ---
AtMPK20         KMY 606
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