The MKK2 Pathway Mediates Cold and Salt Stress Signaling in *Arabidopsis*

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Summary

The *Arabidopsis* mitogen-activated protein kinase (MAPK) kinase 2 (MKK2) and the downstream MAPKs MPK4 and MPK6 were isolated by functional complementation of osmosensitive yeast mutants. In *Arabidopsis* protoplasts, MKK2 was specifically activated by cold and salt stress and by the stress-induced MAPK kinase kinase MEKK1. Yeast two-hybrid, in vitro, and in vivo protein kinase assays revealed that MKK2 directly targets MPK4 and MPK6. Accordingly, plants overexpressing MKK2 exhibited constitutive MPK4 and MPK6 activity, constitutively upregulated expression of stress-induced marker genes, and increased freezing and salt tolerance. In contrast, *mkk2* null plants were impaired in MPK4 and MPK6 activation and were hypersensitive to salt and cold stress. Full genome transcriptome analysis of *MKK2*-overexpressing plants demonstrated altered expression of 152 genes involved in transcriptional regulation, signal transduction, cellular defense, and stress metabolism. These data identify a MAP kinase signaling cascade mediating cold and salt stress tolerance in plants.

Introduction

Like any other living organism, plants have developed sophisticated signaling machineries to adapt their cellular metabolism to a changing environment. In fact, due to their sessile life cycle, plants must respond and protect themselves from all forms of environmental biotic and abiotic stress. A common mechanism to translate such external stimuli into cellular responses is the activation of *m*itogen-*a*ctivated *p*rotein *k*inase (MAPK) cascades. These protein kinase cascades are highly conserved among eukaryotes and consist of three subsequently acting protein kinases, a MAP kinase kinase kinase (MAPKKK), a MAP kinase kinase (MAPKK), and finally the MAP kinase (MAPK). Different MAPK pathways respond to a variety of external stimuli and have been characterized in yeast, animals, and plants (Davis, 2000; Hohmann, 2002; Jonak et al., 2002).

The genome of the yeast *Saccharomyces cerevisiae* encodes six different MAPKs, and cellular functions for five of these MAPKs have been established (Herskowitz, 1995; Gustin et al., 1998; Hohmann, 2002; O'Rourke et al., 2002). In contrast, plants have more than 20 MAPKs, but relatively little is known about the function and composition of the different pathways (Jonak et al., 2002; MAPK Group, 2002; Tena et al., 2001; Zhang and Klessig, 2001). The MAPKs investigated so far were mainly involved in stress responses (Jonak et al., 2002), and in *Arabidopsis*, MPK3, MPK4, and MPK6 are activated by a diverse set of stresses, including pathogens, and osmotic, cold, and oxidative stress (Asai et al., 2002; Desikan et al., 2001; Droillard et al., 2002; Ichimura et al., 2000; Kovtun et al., 2000; Nühse et al., 2000; Petersen et al., 2000). Because the kinetics and magnitude of activation differ between the different MAP kinases for different stresses, it is likely that different MAPKs play different roles in these stress responses.

Compared to our current knowledge of plant MAPKs, much less is known about the functions of their upstream activators or of the complete signaling pathways. From a total of ∼1000 protein kinases in *Arabidopsis*, more than 100 genes encode MAPK pathway components. Interestingly, the *Arabidopsis* genome features more than 60 putative MAPKKKs yet only 20 MAPKs and 10 MAPKKs (*Arabidopsis* Genome Initiative, 2000; MAPK Group, 2002). These numbers suggest that the 10 MAPKKs are major, multifunctional entry routes for upstream signal integration as well as bifurcation points for activation of downstream MAPKs. The activation of different downstream MPKs by one particular MAPKK was reported in *Medicago* and tobacco (Cardinale et al., 2002; Jin et al., 2003, Ouaked et al., 2003). In *Arabidopsis*, MKK4 and MKK5 can activate both MPK3 and MPK6 (Asai et al., 2002), whereas two-hybrid assays and yeast complementation suggested that MPK4 can also be activated by MKK1 (Mizoguchi et al., 1998; Huang et al., 2000; Matsuoka et al., 2002). Matsuoka et al. (2002) also showed that MKK1 is rapidly activated in response to wounding in *Arabidopsis* seedlings. So far, no genetic or biochemical function has been ascribed to the *Arabidopsis* MAPKK MKK2.

We describe here the functional characterization of MKK2. Biochemical and genetic analysis indicates that MKK2 plays a critical role in the cold and salt stress

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response in *Arabidopsis*. Full genome transcriptome analysis of plants expressing active MKK2 revealed a significant change in expression of 152 genes encoding proteins involved in transcriptional regulation, defense, signaling, and metabolism, many of which overlap with sets of recently identified marker genes for cold and salt stress response in *Arabidopsis* (Fowler and Thomashow, 2002; Kreps et al., 2002; Seki et al., 2002). Our data suggest that MKK2 is part of a signal transduction module consisting of the MAPKKK MEKK1 as upstream activator of MKK2 and the downstream MAPKs MPK4 and MPK6.

Results

The *Arabidopsis* MAPKK MKK2 was isolated by functional complementation of the osmosensitive yeast strain *pbs2Δ*, lacking the MAPKK of the HOG1 pathway (data not shown). To isolate potential downstream MPKs of MKK2, we used a *pbs2Δ hog1Δ* double mutant expressing *Arabidopsis* MKK2. In this screen, we isolated only MPK6 but no other MAPK. To test the specific requirement for MPK6 in this functional complementation, we tested the most closely related *Arabidopsis* MAPKs MPK4 and MPK3, which are also stress activated (Ichimura et al., 2000; Kovtun et al., 2000), by cotransforming a *pbs2Δ hog1Δ* strain with these MAPKs and MKK2. MPK6, and to a lower degree MPK4, complemented the pbs2Δ hog1Δ mutant, but only in the presence of MKK2 (Figure 1A). In contrast, MKK2 could not complement the pbs2Δ hog1Δ mutant when coexpressed with MPK3 (Figure 1A). To test whether the closely related MAPKK MKK1 can complement pbs2Δ hog1Δ mutant yeast, MPK3, MPK4 and MPK6 were also coexpressed with MKK1 in this strain. However, no complementation was obtained in any of the three combinations (see Supplemental Figure S1A at http://www. molecule.org/cgi/content/full/15/1/141/DC1).

To test for direct interaction of different MPKs with MKK2, we cloned twelve *Arabidopsis* MAPKs, representing all MAPK subfamilies into the GAL4 activation domain vector pGAD and measured their interaction with MKK2 in quantitative β-galactosidase assays. MKK2 showed strongest interaction with MPK4 and MPK6 and to a much lower degree also with MPK5 (Figure 1B). For comparison, we also tested MKK1 as the most closely related MAPKK of MKK2 in *Arabidopsis*. In contrast to MKK2, MKK1 interacted selectively with MPK4 (Supplemental Figure S1B).

MKK2 Activates MPK4 and MPK6 by Phosphorylation

To investigate whether MKK2 can phosphorylate MPK4 and MPK6, we expressed and purified recombinant kinase inactive GST fusion proteins of MPK3, MPK4, and MPK6. MKK2 was immunoprecipitated from transiently transformed protoplasts under control of the 35S Cauliflower mosaic virus (CaMV) promoter and tested for its ability to phosphorylate MPK3, MPK4, and MPK6 in vitro after activation by cold stress for 10 min at 4°C. As shown in Figure 1C, MPK4 and MPK6 were phosphorylated by MKK2 but not MPK3. As a control, recombinant MPK3, MPK4, and MPK6 were also tested for phosphorylation by MKK1. For this purpose, MKK1 was similarly ex-
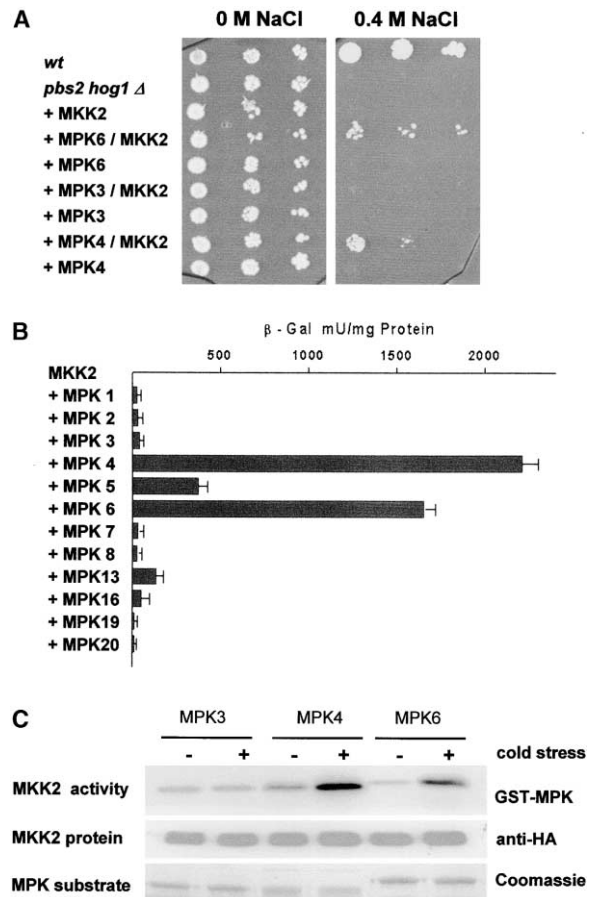

Figure 1. Complementation of Yeast Mutants, Specificity of MKK2-MAP Kinase Interactions, and Substrate Specificity of MKK2 In Vitro

(A) Complementation of osmosensitive *pbs2Δ hog1Δ* mutants by MKK2/MPK6 and MKK2/MPK4, but not MKK2/MPK3. MPK6, MPK3, or MPK4 constructs alone or in combination with the *Arabidopsis* MKK2 were transformed in the *pbs2Δ hog1Δ* strain, and growth on salt media was compared to the parental wild-type strain W303 (wt) as described in the Experimental Procedures. A loading control is shown in the left panel (0 M NaCl).

(B) Quantitative yeast two-hybrid analysis of pBTM116-MKK2 with different pGAD424-MPKs, representing all MAPK subfamilies in the *Arabidopsis* genome.

(C) In vitro phosphorylation of MPK3, MPK4, and MPK6 by active MKK2. HA epitope-tagged MKK2 was immunoprecipitated from *Arabidopsis* protoplasts before and 10 min after cold stress treatment. Immunoprecipitated MKK2 was subsequently used for phosphorylation of recombinant kinase inactive GST-MPK3, GST-MPK4, and GST-MPK6, respectively. Phosphorylation of MPKs was analyzed by autoradiography after SDS-PAGE. MKK2 protein was detected using HA antibodies, and a Coomassie stain of the MPK substrates is shown in the lower panel.

pressed in protoplasts but this time activated by H_2O_2 as cold did not activate MKK1 (see below). Whereas MPK4 was phosphorylated by MKK1, no increase in MPK3 or MPK6 phosphorylation could be detected (Supplemental Figure S1C).

To verify this result under more natural conditions, we tested the activation of different MPKs by MKK2 in *Arabidopsis* protoplasts using a constitutively active MKK2 allele (MKK2-EE). For this purpose, protoplasts were cotransformed with MYC epitope-tagged wild-type (MKK2-wt) or constitutively active MKK2 (MKK2-
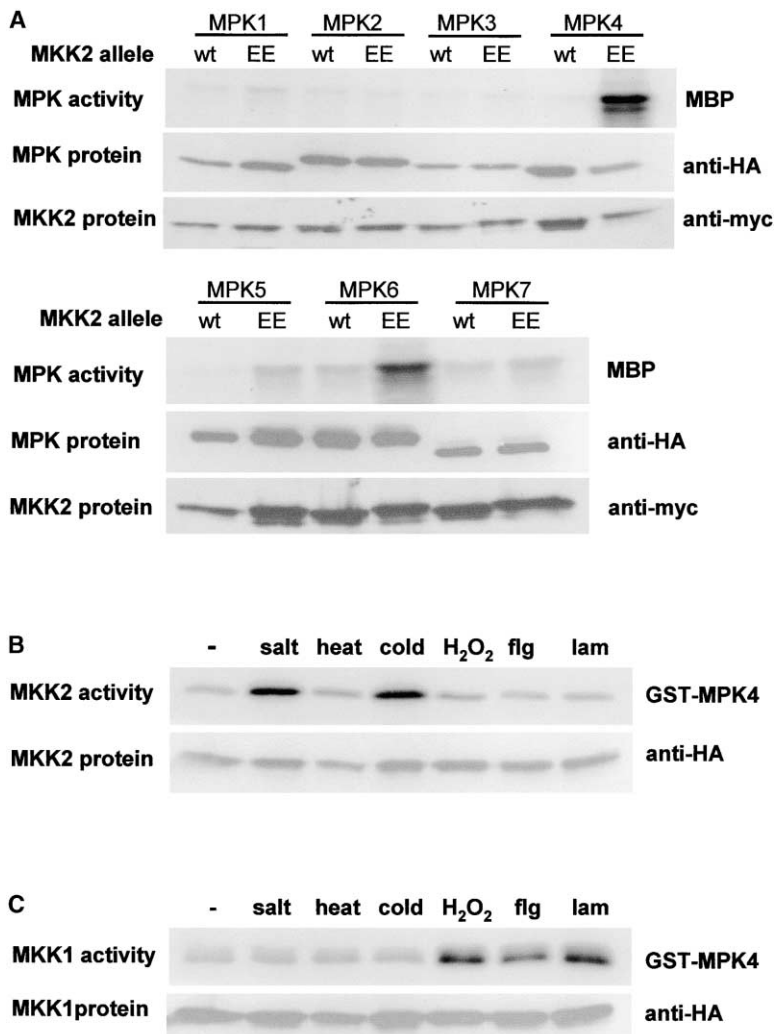
A

B

C

Figure 2. Activation of Downstream MAP Kinases by MKK2, and Stress-Induced Activation of MKKs in *Arabidposis* Protoplasts

(A) Activation of different MPKs by MKK2. Activation of MPKs was tested by coexpression of MPKs with either wild-type MKK2 (wt) or constitutively active MKK2 (EE). Kinase activity of immunoprecipitated MPKs was measured with MBP as artificial substrate in in vitro kinase assays. Phosphorylation of MBP was detected by autoradiography after SDS-PAGE. Expression of the MPKs and MKK2 (lower panels) was detected by Western blot analysis with HA (MPKs) or c-MYC antibody (MKK2).

(B) Activation of MKK2 by salt and cold stress. MKK2 activity was determined after transient expression in plant cells upon different stress treatments. HA epitope-tagged MKK2 was immunoprecipitated from *Arabidopsis* protoplasts following stress treatments for 10 min: salt (280 mM NaCl), heat (37°C), cold (0°C), H_2O_2 (2 mM), flg (10 nM flagellin), and lam (10 μg/ml laminarin). MKK2 kinase activity was determined by in vitro kinase assays using kinase-inactive GST-MPK4 as a substrate.

(C) Activation of MKK1 by H_2O_2, flagellin, and laminarin. MKK1 activity was determined after transient expression in plant cells upon the different stress treatments as described above for (B). HA epitope-tagged MKK1 was immunoprecipitated from *Arabidopsis* protoplasts, and kinase activity was determined by in vitro kinase assays using kinase-inactive GST-MPK4 as a substrate.

EE) in the presence of different HA epitope-tagged MPKs. After immunoprecipitation of the MPKs, activation of the MAPKs was determined by in vitro kinase assays with myelin basic protein (MBP) as artificial substrate. Whereas little activation of MPKs was seen upon coexpression with wild-type MKK2, MPK4 and MPK6 were strongly activated by constitutively active MKK2 (Figure 2A). Consistent with the much weaker yeast two-hybrid interaction (Figure 1B), MPK5 was also activated to a much lesser extent in these assays. As shown by protein gel blot analysis for MPK proteins (anti-HA antibody) and for MKK2 (anti-myc antibody) (Figures 2B and 2C, lower panels), different expression levels of MKK2 and MPKs could not account for the observed differences in activation levels. These results show that MKK2 has the potential to activate MPK4, MPK5, and MPK6.

Salt and Cold Stress Activation of MPK4 and MPK6 Is Mediated by MKK2

To address the question of which stimuli might trigger the MKK2 pathway in plant cells, we expressed HA epitope-tagged MKK2 and MKK1 in protoplasts and measured phosphorylation of recombinant kinase-inactive GST-MPK4 as a common substrate following different stress treatments. As shown in Figure 2B, MKK2 was

activated by salt and cold stress, but not by heat, hydrogen peroxide, the flagellin-derived bacterial peptide elicitor flg22 or laminarin (lam), a β-glucan. In contrast, MKK1 was not activated by salt, cold, and heat stress conditions, but by hydrogen peroxide, flg22, and laminarin (Figure 2C). These data indicate that MKK2 is activated in vivo in response to salt and cold stress.

mkk2 Null and MKK2-Overexpressing Plants Exhibit Opposite Cold and Salt Tolerance Phenotypes

We obtained an *MKK2* T-DNA insertion line from the Syngenta *Arabidopsis* Insertion Library. This line carries a single T-DNA insertion in intron 5 of the *MKK2* gene (Figure 3A; Experimental Procedures), leading to an mRNA null phenotype (Figure 3B). We also generated *MKK2*-overexpressing lines in the Columbia (Col-0) background using either wild-type *MKK2-WT* or constitutively active *MKK2-EE* genes expressed as MYC-epitope-tagged versions under control of the constitutive 35S CaMV promoter.

mkk2 null and *MKK2* overexpressor plants had no obvious phenotype under normal ambient conditions but showed strong differences upon cold and salt stress treatments. As shown in Figure 3C, *MKK2-EE*-overexpressing plants displayed increased freezing tolerance

A

B

Col-0   *mkk2*

MKK2

ACT3

C

- stress   accl.   shock

Col-0

mkk2

MKK2
EE4

D

Col-0   *mkk2*

- NaCl

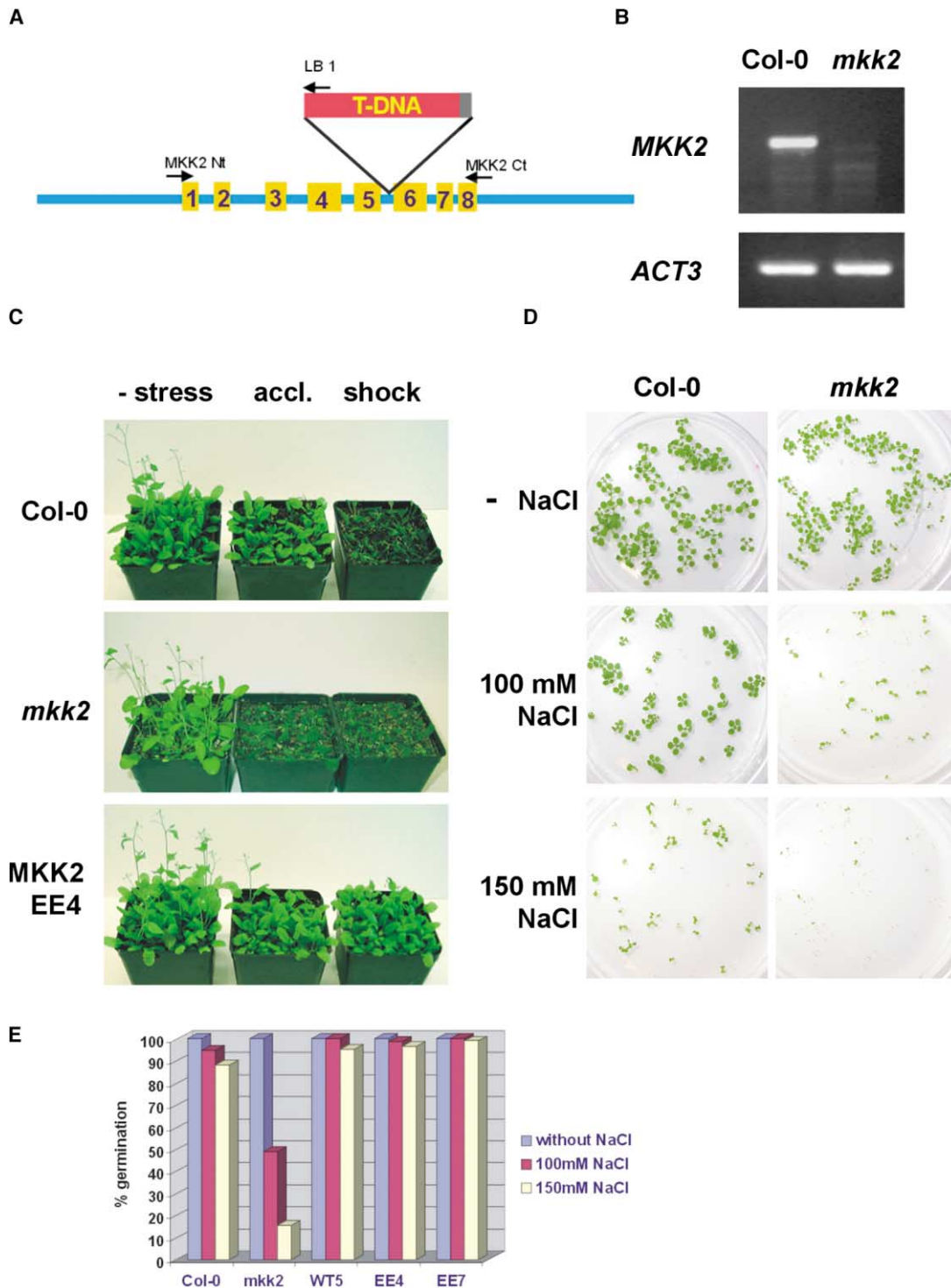100 mM
NaCl

150 mM
NaCl

E

Figure 3. Phenotypic Analysis of *mkk2* Null and Overexpressing Lines

(A) Intron-exon structure of the *MKK2* gene according to the Plants P database (http://plantsp.sdsc.edu/) and the position of the T-DNA insertion, determined by PCR and sequencing of the flanking regions, as described in the Experimental Procedures.

(B) *MKK2* transcript levels in wild-type Col-0 plants and the *mkk2* null line (*mkk2*) analyzed by RT-PCR from leaves of 3-week-old plants.

(C) Freezing sensitive phenotype of *mkk2* null plants and freezing tolerance of the *MKK2*-overexpressing line EE4. Freezing treatment was carried out as described in the Experimental Procedures either with previous acclimation (accl.) for 24 hr at +4°C or by directly subjecting the plants to freezing conditions (shock). Pictures were taken 3 days after freezing treatment.

(D) Salt-sensitive phenotype of *mkk2* null lines. Germination of wild-type (Col-0) and *mkk2* null plants (*mkk2*) was tested on agar plates without and with 100 mM or 150 mM NaCl. Germination was visually scored 10 days after putting plants into the light as described in the Experimental Procedures.

(E) Statistical analysis of germination of wild-type plants (Col-0), the *mkk2* null line, and the *MKK2*-overexpressing lines WT5, EE4, and EE5 (EE lines are overexpressing constitutively active MKK2) on salt-containing media.

A   Col-0

	0	15	30	60	2h	4h	8h	24h	at +4°C
MPK4 activity									MBP
MPK4 protein									anti-MPK4
MPK6 activity									MBP
MPK6 protein									anti-MPK6
Rubisco LSU									Ponceau

B   *mkk2*

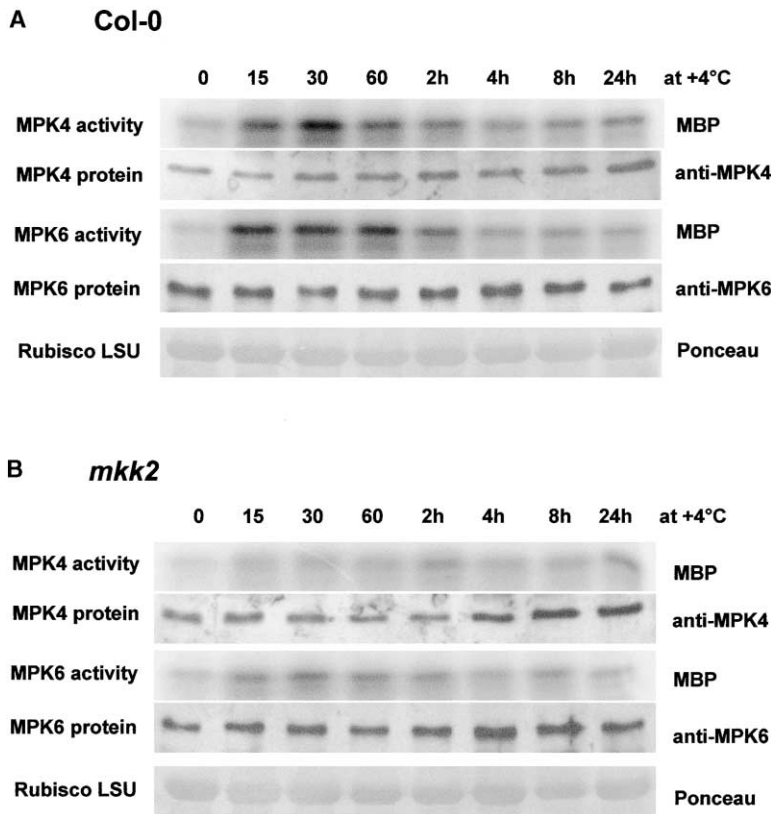|	0	15	30	60	2h	4h	8h	24h	at +4°C
MPK4 activity									MBP
MPK4 protein									anti-MPK4
MPK6 activity									MBP
MPK6 protein									anti-MPK6
Rubisco LSU									Ponceau

Figure 4. Cold-Triggered Activation of MPK4 and MPK6 in Planta

Kinetics of MPK4 and MPK6 activation was measured in wild-type (A) and *mkk2* null plants (B) in response to cold stress. MPK4 and MPK6 were immunoprecipitated from leaf cell extracts of cold-shocked plants. MPK activity was measured in immunocomplex kinase assays using MBP as substrate, and levels of MPK4 and MPK6 proteins were detected in Western blots. As additional loading control, the large subunit of Rubisco (LSU) is shown after Western blotting.

compared to wild-type or *mkk2* null plants. While wild-type and *MKK2-EE*-overexpressing plants survived freezing conditions after cold acclimation, *mkk2* null plants were hypersensitive to cold stress. We obtained similar results with nine different *MKK2*-overexpressing lines in at least three independent assays.

To analyze salt tolerance of the *MKK2*-overexpressing lines and the *mkk2* null plants, we analyzed the ability of these lines to germinate on salt-containing media. Compared to wild-type Col-0, *mkk2* null plants were strongly compromised to germinate on salt-containing media (Figure 3D). In contrast, *MKK2*-overexpressing lines showed slightly improved ability to germinate on salt-containing media (data not shown). A quantification of the differences in the germination ability of the different lines on salt-containing media confirmed the qualitative analysis (Figure 3E), showing that the *MKK2* gene is important for conferring salt stress tolerance.

mkk2 Null Plants Are Impaired in Cold-Responsive MAP Kinase Activation

To investigate the molecular mechanism of MKK2 action in the cold stress response, MPK4 and MPK6 activation was analyzed in wild-type Col-0 and *mkk2* null plants. For this purpose, endogenous kinase activities of MPK4 and MPK6 were determined after cold treatment using immunocomplex kinase assays. Endogenous MPK4 or MPK6 was immunoprecipitated from leaf protein extracts with antibodies directed against MPK4 or MPK6. In vitro kinase assays with MBP as substrate revealed that MPK4 and MPK6 were both activated by cold treatment at 15 min in wild-type Col-0 plants (Figure 4A). In

the *mkk2* null line, cold-induced activation of MPK4 was almost undetectable, whereas MPK6 activation was significantly reduced, but not completely abrogated (Figure 4B). These data suggest that MKK2 is more important for cold-induced activation of MPK4 than for MPK6, consistent with the stronger interaction of MPK4 with MKK2 in yeast two-hybrid assays (Figure 1B) and the stronger activation of MPK4 by MKK2 in vitro and in vivo (Figures 1C and 2A). Protein levels of MPK4 and MPK6 remained constant throughout cold treatment, confirming that activation of MPK4 and MPK6 occurs primarily by posttranslational modification.

Enhanced Expression of Cold and Salt Stress Marker Genes in *MKK2* Overexpression Lines

To clarify whether *MKK2* functions in transcriptional regulation of cold and salt stress genes, we compared MKK2 kinase activities with transcript levels of several known stress marker genes. MKK2 kinase activity in the overexpressor lines was determined by immunocomplex kinase assays using a MYC-specific antibody for precipitation of MKK2 and recombinant kinase-inactive GST-MPK4 as substrate (Figure 5A). Although some differences were observed between different *MKK2* overexpressor lines, in general, lines overexpressing constitutively active *MKK2 (*MKK2-EE*)* had higher MKK2 kinase activity compared to lines overexpressing wild-type *MKK2* (MKK2-WT).

We next performed RT-PCR analysis for seven genes known to be upregulated upon cold and salt stress treatment (Gilmour et al., 1998; Fowler and Thomashow, 2002; Kreps et al., 2002) and actin as control (Figure 5B).
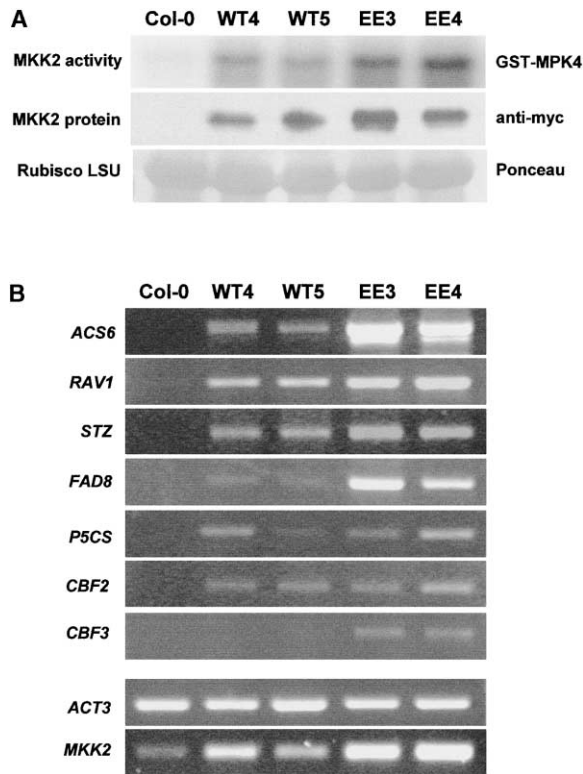
A

B

Figure 5. Basal Levels of MKK2 Activities and Marker Gene Expression in *MKK2*-Overexpressing Lines

(A) MKK2 kinase activities in different *MKK2* overexpressor lines. Protein extracts were prepared from wild-type (Col-0) and different wild-type (WT4 and WT5) and constitutively active MKK2 (EE3 and EE4) lines. MKK2 was immunoprecipitated using MYC antibody. MKK2 activity was determined by in vitro kinase assays using kinase-inactive GST-MPK4 as a substrate. MKK2 protein was detected using MYC antibody, and the large subunit of Rubisco (LSU) is shown as additional control for equal protein amounts.

(B) Expression of marker genes for cold and salt stress response in different *MKK2*-overexpressing lines as detected by RT-PCR. Total RNA was prepared as described in the Experimental Procedures. Marker gene fragments were amplified using gene-specific primers. The *ACT3* gene coding for actin was used as control for equal loading.

RAV1, *STZ*, *CBF2*, and CBF3 encode four transcription factors that are strongly induced within 30 min after cold and salt stress in *Arabidopsis* (Fowler and Thomashow, 2002). Compared to wild-type plants, the four transcription factors were upregulated in the *MKK2*-overexpressing lines under nonstress conditions. *ACS6*, encoding aminocyclo-propane-1-carboxylic acid (ACC) synthase; *FAD8*, encoding a chloroplast localized fatty acid desaturase, and *P5CS*, encoding a key enzyme in proline biosynthesis, also exhibited increased expression levels in the *MKK2*-overexpressing lines. Overall, plants overexpressing the constitutively active *MKK2* allele showed higher expression levels of the seven selected stress marker genes than lines overexpressing wild-type *MKK2*. These data show that ectopic MKK2 activity is correlated with upregulation of salt and cold stress marker genes.

MKK2 Regulates Expression of 152 Genes

To define the total regulon of genes controlled by *MKK2*, DNA microarray analysis was carried out on two *MKK2*-overexpressing lines, *MKK2-WT4* and *MKK2-EE4* under normal growth conditions. After preparation of total RNA from these lines and wild-type plants, transcriptome analysis was performed using the *Arabidopsis* ATH1 Genome Array from Affymetrix (Experimental Procedures), representing ∼24,000 *Arabidopsis* genes. Gene expression ratios were calculated, comparing expression levels in MKK2-WT4 and MKK2-EE4 to those of Col-0 wild-type plants. A final set of 152 genes showing at least 3-fold or greater expression difference in the two comparisons was selected and subjected to hierarchical clustering (Figure 6A). These genes were divided into two groups according to their expression characteristics: Cluster I represents the 127 genes that are upregulated at least 3-fold in the *MKK2*-overexpressing lines, while the 25 cluster II genes exhibit reduced expression levels in the *MKK2*-overexpressing lines relative to wild-type plants (Figure 6A). In general, when compared to *MKK2*-WT wild-type overexpressor lines, the *MKK2-EE* lines had a stronger quantitative effect on the upregulation of most of the genes that were identified.

Discussion

MKK2 Mediates Cold and Salt Stress Responses

MAPK pathways mediate cellular responses to a great variety of different extracellular signals in plants. In this work, we have analyzed the function of the MAPKK MKK2. Of central importance in attributing a function to a given MAPKK are consistent genetic and biochemical data in the context of a signaling pathway. By using transient expression assays in protoplasts, we demonstrated that MKK2 is activated in response to cold and salt stress. A reverse genetic approach proved that overexpression of wild-type or constitutively active *MKK2* resulted in elevated MAPK kinase activity of MKK2 and enhanced freezing and salt tolerance. In contrast, *mkk2* null mutant plants exhibited hypersensitivity to freezing and germination on salt-containing media. These data demonstrate that MKK2 is a key signal transducer of cold and salt stress in *Arabidopsis*.

MPK4 and MPK6 Are Direct Downstream Targets of MKK2

The *Arabidopsis* genome contains 10 MAPKKs and at least 20 MAPKs, suggesting that any particular MAPKK should be able to activate more than one downstream MAPK. Activation of multiple MAPKs by MAPKKs has been demonstrated in *Medicago*, tobacco, and *Arabidopsis* (Asai et al., 2002; Cardinale et al., 2002; Yang et al., 2001; Jin et al., 2003). Therefore, the identification of the downstream targets of a given MAPKK is of major importance. We approached this problem in several ways. By yeast two-hybrid analysis, MPK4 and MPK6 were identified as the strongest interactors with MKK2. We confirmed the importance of these interactions by showing that MPK4 and MPK6 are specifically phosphorylated and activated by MKK2 both in vitro and in vivo. These results demonstrated that MPK4 and MPK6
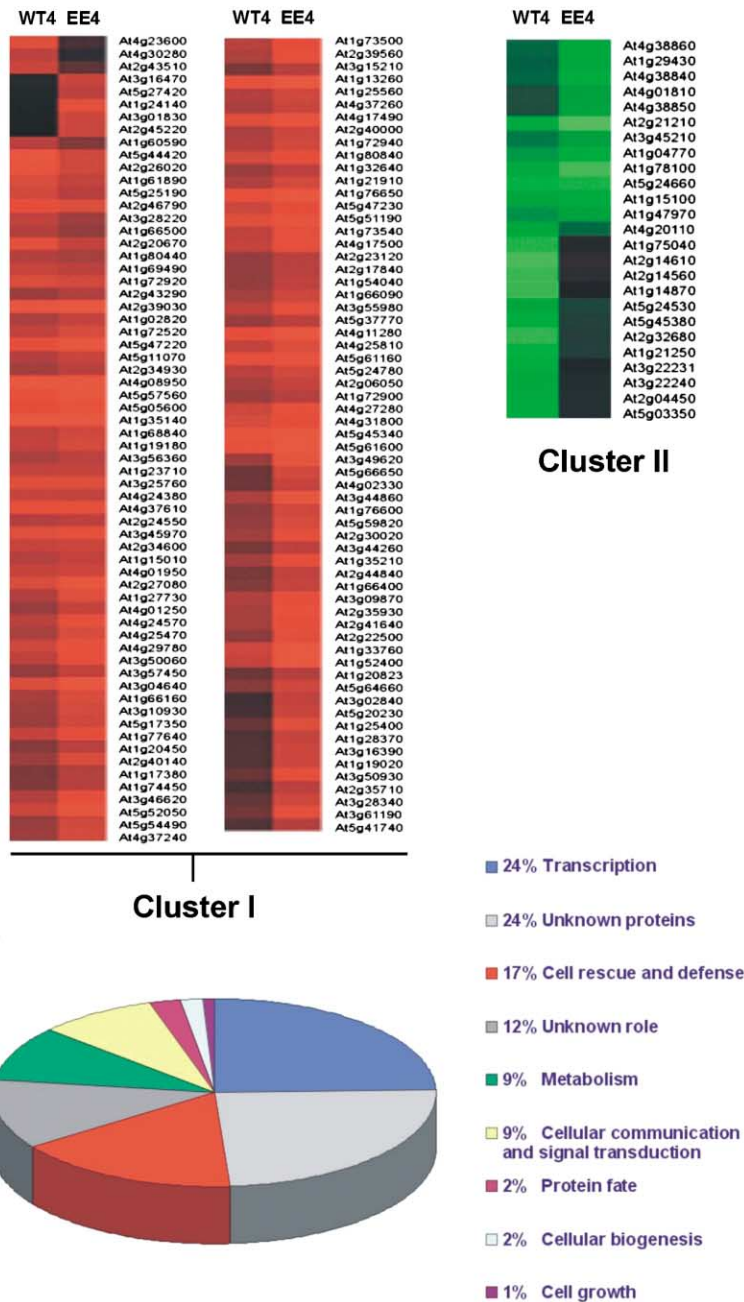
A

Cluster I

Cluster II

B

Figure 6. Profiling of MKK2 Triggered Gene Expression Responses

(A) Hierarchical clustergram of 152 genes that show at least > or = 3-fold expression difference from wild-type Col-0 plants in the absence of stress treatments. The 152 affected genes were divided into two clusters: Cluster I represents upregulated genes, and cluster II represents downregulated genes. Clustering was performed using Cluster and TreeView (Eisen et al., 1998). Expression analysis was done using *Arabidopsis* ATH1 Genome arrays from Affymetrix. Further information about the 152 affected genes, including the gene identifiers, is provided in Table 1 and the Supplemental Data.

(B) Functional classification of upregulated genes in the *MKK2*-overexpressing lines.

- 24% Transcription
- 24% Unknown proteins
- 17% Cell rescue and defense
- 12% Unknown role
- 9% Metabolism
- 9% Cellular communication and signal transduction
- 2% Protein fate
- 2% Cellular biogenesis
- 1% Cell growth

are direct targets of MKK2. In contrast to the more closely related MAPKK gene pair MKK4 and MKK5 (75% identity at the amino acid level), which seems to mediate flagellin-induced MPK3 and MPK6 activation (Asai et al., 2002), the less related MAPKK pair of MKK1 and MKK2 (62% identity) seems to function in different pathways. Although both MKK1 and MKK2 were found to interact, phosphorylate, and activate MPK4, only MKK2 was able to target also MPK6 (Figure 1C and see Supplemental Figure S1C at http://www.molecule.org/cgi/content/full/15/1/141/DC1). Activation assays showed that MKK1

and MKK2 are activated by different external factors. Whereas MKK2 responded most strongly to cold and salt stress (Figure 2B), MKK1 showed highest activation by H_2O_2 as well as by a bacterial and fungal elicitor (Figure 2C). At a first glance, these results seem to contradict the findings of Matsuoka et al. (2002) who reported that MKK1 can be activated by wounding, cold, drought, and high salt. However, comparing the levels and kinetics of MKK1 activation as reported by Matsuoka et al., it turns out that MKK1 activity showed by far the strongest and fastest activation in response to
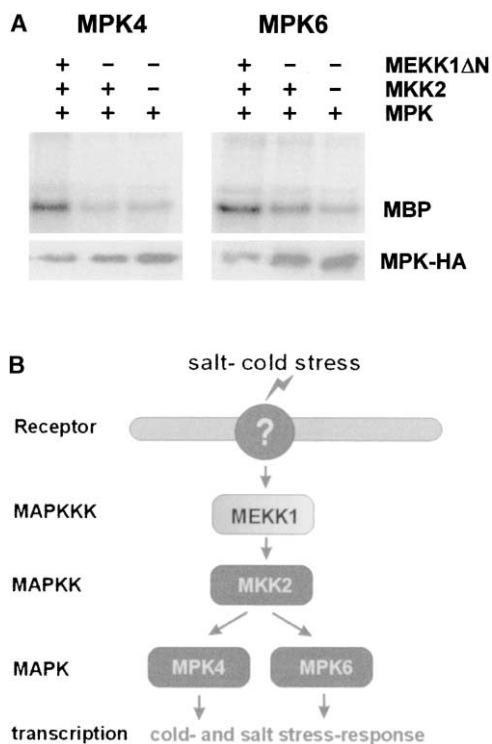
Figure 7. Model of the MKK2 Pathway

(A) MPK4 and MPK6 activation by MEKK1 is mediated by MKK2. Activation of MPK4 and MPK6 was tested either by expression of the MPKs alone, or together with MKK2, in the absence and presence of MEKK1. Kinase activity of immunoprecipitated MPKs was measured by in vitro kinase assays using MBP as artificial substrate. Phosphorylation of MBP was detected by autoradiography after SDS-PAGE. Expression of the MPKs was detected by Western blot analysis with HA antibody.

(B) Proposed working model for the cold and salt stress-triggered MKK2 pathway involving MEKK1 as an upstream activator of MKK2 and the downstream MAPKs MPK4 and MPK6.

wounding, which is consistent with our observation that MKK1 is activated by H_2O_2 and elicitors. Furthermore, the discrepancy could also be due to the use of different experimental systems (seedlings versus protoplasts) and to the peptide sequence Matsuoka et al. have used to raise the anti-MKK1 antibody, which is in part completely identical to that of the MKK2 C terminus.

MEKK1 Can Function as an Upstream Regulator of MKK2

The MEKK1 gene was identified to be upregulated by cold and salt stress (Mizoguchi et al., 1996). Furthermore, by yeast two-hybrid assays, MEKK1 was shown to interact with MKK2 (Mizoguchi et al., 1998; Ichimura et al., 1998). Therefore, MEKK1 was tested as a potential upstream activator of MKK2 in protoplast assays. Whereas expression of MPK4 or MPK6 alone or in combination with wild-type MKK2 resulted in poor activation of the MAPKs, coexpression of constitutively active N-terminally truncated MEKK1 (MEKK1ΔN) together with MKK2 and MPK4 or MPK6 resulted in strong activation of either MAPK (Figure 7A). These results suggest that MEKK1-MKK2-MPK6/MPK4 can form a MAPK module

and that MEKK1 is an upstream activator of MKK2 (Figure 7B).

The MKK2 Pathway Affects Expression of 152 Genes

The responses to abiotic stresses can generally be separated into two categories: those that regulate signal transduction and gene expression in response to the stress, and those that protect against environmental stress by redirection of metabolism. Resistance to cold and salt stress requires a number of changes in gene expression (Fowler and Thomashow, 2002; Zhu, 2001; Xiong et al., 2002).

An overall analysis of the transcriptome profile of MKK2 overexpressor lines revealed that the MKK2 regulon was comprised of 152 genes (Figure 6A). For a more detailed analysis, the gene set was subdivided into seven functional categories (Figure 6B). The upregulated genes encode proteins involved in transcription, signaling, cell rescue and defense, and metabolism, whereas the downregulated genes are mostly related to auxin-regulated transcription (Table 1). A complete list of all identified genes is shown in the Supplemental Data. In agreement with previous transcriptome analyses of plant stress (Fowler and Thomashow, 2002; Kreps et al., 2002; Seki et al., 2002), multiple types of transcription factors constitute the largest group of the upregulated genes (cluster I: 31 of all 127 upregulated genes, p = 7.4 E-11). Besides the typical abiotic stress marker genes RAV1,2 DREB1C/CBF2 and STZ, several known MPK6 targets were also identified including several ethylene-response element binding factors (ERFs) and WRKY transcription factors.

Of the signal transduction elements, several calmodulins and calcium binding proteins were strongly upregulated in the MKK2 overexpressor lines (Table 1). Interestingly, the MAPKK MKK5 also showed enhanced expression levels. MKK5 is an activator of MPK3 and MPK6 in response to the bacterial elicitor flagellin (Asai et al., 2002). Upregulation of MKK5 in the MKK2 overexpressor lines could indicate some level of crosstalk between these pathways. Because some protein phosphatase 2Cs (PP2Cs) are negative regulators of MAPK pathways (Meskiene et al., 2003), it could be possible that the increased expression levels of a PP2C could be part of a negative feedback mechanism.

Among the genes involved in cell rescue and defense, several enzymes and targets of the ethylene and jasmonate pathways were upregulated (Table 1). Whereas MPK6 is involved in ethylene signaling and biosynthesis (Kim et al., 2003; Ouaked et al., 2003), MPK4 is implicated in jasmonate signaling (Petersen et al., 2000), suggesting that the MKK2 pathway may integrate the synthesis and signaling of these two hormones in the abiotic stress response. Besides TCH4, encoding an xyloglucan endotransglycosylase, several enzymes of the secondary metabolism showed augmented expression (Table 1).

When the transcriptome profiles of the MKK2 overexpressor lines were analyzed for downregulated genes (cluster II), almost only factors involved in auxin-regulated gene expression were identified (Table 1). These data suggest that the MKK2 pathway might be involved in negatively regulating certain aspects of auxin signal-

Table 1. Prominent Up- and Downregulated Genes

Putative Function and Reference	AGI#	Ratio WT4/Col-0	Ratio EE4/Col-0
Upregulated Genes			
Transcription Factors:			
DNA binding protein (RAV1)[a,b]	At1g13260	6.48	10.41
Similar to DNA binding protein RAV2	At1g25560	3.09	4.03
DRE binding protein (DREB1C); identical to CBF2[a,c]	At4g25470	2.87	4.23
AP2 domain protein RAP2.8 (RAV2); identical to RAV2[a]	At1g68840	3.38	3.92
Salt tolerance zinc finger protein (STZ)[a,d]	At1g27730	3.01	4.59
Zinc finger protein Zat12[c,d]	At5g59820	2.45	4.18
Ethylene-responsive element binding factor(AtERF6)[c,d]	At4g17490	4.63	6.98
WRKY family transcription factor	At1g80840	4.12	5.91
Myb DNA binding protein	At3g50060	3.30	5.16
WRKY family transcription factor	At4g31800	4.19	5.90
Signal Transduction:			
Similar to MAPKK5	At1g73500	3.23	5.67
Protein phosphatase 2C	At2g30020	2.69	4.88
Putative calmodulin	At1g76650	6.08	9.54
Cell Rescue and Defense:			
Defensin PDF1.2a	At5g44420	7.40	3.93
Disease resistance protein (TIR_NBS)	At1g72920	4.38	4.02
Integral membrane protein-like	At5g52050	3.75	7.12
Lipoxygenase	At1g72520	3.42	4.18
ACC synthase (AtACS-6)[a,c,d]	At4g11280	5.43	7.85
Metabolism:			
Xyloglucan endotransglycosylase (TCH4)	At5g57560	6.43	7.63
Flavonol synthase	At5g05600	5.51	6.63
Anthocyanin 5-aromatic acyltransferase	At5g61160	5.01	6.89
Oxidoreductase	At3g49620	2.63	7.04
Downregulated Genes			
Putative auxin-induced protein[b,c]	At4g38860	0.52	0.30
Auxin-induced protein	At1g29430	0.55	0.31
Auxin-induced protein[b,c]	At4g38840	0.52	0.27
Small auxin up RNA (SAUR-AC1)[b]	At4g38850	0.65	0.32
Putative auxin-regulated protein[b,c]	At2g21210	0.29	0.15

[a] Fowler and Thomashow, 2002.
[b] Cheong et al., 2002.
[c] Kreps et al., 2002.
[d] Seki et al., 2002.

ing. Possibly, this effect is mediated by MPK6 which together with MPK3 has been shown to block auxin signaling (Kovtun et al., 2000), but further analysis of the implicated MAPKs are necessary to clarify this issue. Hence, the transcriptome analysis places MKK2, and thereby MPK4 and MPK6, squarely into a signaling regulon that contains a high degree of correspondence to previous stress transcriptome analyses (Fowler and Thomashow, 2002; Kreps et al., 2002; Seki et al., 2002).

Taken together, our data define MKK2 as a key signal transducer for cold and salt stress. Biochemical and genetic analysis revealed that MKK2 modulates cold and salt tolerance through expression of a set of 152 genes. In vitro and in vivo evidence suggests that MKK2 mediates stress signaling by a MAPKKK-MAPKK-MAPK module composed of MEKK1-MKK2-MPK4/MPK6 (Figure 7B). The identification of the MKK2 pathway lays the ground for a molecular understanding and improving cold and salt stress tolerance in crop plants. However, a number of important questions still remain to be addressed in the future such as the identification of the cold and salt stress sensors that activate the MKK2 cascade and the exact mechanism how the set of 152 genes is regulated by the MKK2 pathway.

Experimental Procedures

Arabidopsis ecotype Columbia (Col-0) was used as genetic background. Seeds were germinated on 0.5× MS medium (Sigma), and plants were grown at 50 μE cool white light under long day conditions (16 hr light/8 hr dark). *Arabidopsis* protoplasts were prepared from a suspension culture as described in Cardinale et al. (2000).

Functional Complementation of Yeast Mutants
The osmosensitive yeast strain *PBS2::HIS3* (Reiser et al., 2000) was used for complementation with an *Arabidopsis* cDNA library (Minet et al., 1992). Positive clones were selected on media containing 400 mM NaCl and analyzed by DNA sequencing. Subsequently, a *hog1*Δ, *pbs2*Δ double null strain was generated (*HOG1::TRP; PBS2::HIS*) and transformed with the *Arabidopsis* MKK2 gene cloned into the yeast expression vector YEp181 (Gietz and Sugino, 1988) before performing a second screen. Complementation of the *hog1*Δ, *pbs2*Δ mutant was done with the open reading frames of MPK4, MPK6, MKK1, and MKK2, cloned in the yeast vectors pRS316 (MPKs) or pRS315 (MKKs) under control of the ADH promoter (Sikorski and Hieter, 1989). Osmosensitivity of yeast strains was tested by spotting serial dilutions of logarithmically growing cells onto selective plates with and without salt.

Yeast Two-Hybrid Assays
Quantitative yeast two-hybrid assays were done as described in Teige et al. (2001) using the yeast two-hybrid strain L40 and the vectors pBTM116 (Vojtek et al., 1993) for the LexA-BD fusions of

the MKKs, and pGAD424 (Clontech, Palo Alto, CA) for the Gal4-AD fusions of the MPKs.

Molecular Cloning and Construction of Expression Vectors
Open reading frames of the different MPKs and MKKs were amplified from a cDNA library (Minet et al., 1992) with an NcoI restriction site at the 5′ end and a NotI restriction site in front of the stop codon. The NotI restriction site was used to introduce either a triple hemagglutinin (HA) epitope or a c-MYC epitope. Gain-of-function (GOF) alleles of MKK2 were generated by changing both putative phosphorylation sites to glutamate residues (T220E and T226E). These point mutations resulted in a constitutively active kinase (MKK2-EE).

Expression and Purification of GST Fusion Proteins
Escherichia coli strain BL-21 codon plus (Stratagene, La Jolla, CA) was transformed with each expression construct, cloned into the pGEX4-T1 vector (Amersham-Pharmacia Biotech, UK). Growth of bacteria and isolation of recombinant GST-fusion proteins were done according to Matsuoka et al. (2002).

Protein Extracts from *Arabidopsis* Protoplasts and Leaves
Protein extracts were either prepared from protoplasts as described in Cardinale et al. (2002) or from 200 mg of frozen leaves in 200 μl Lacus buffer and sea sand according to Bögre et al. (1999).

In Vitro Kinase Assays
The coding regions of MPK3, MPK4, and MPK6 were cloned into pGEX-4T-1 vector and expressed as glutathione S-transferase (GST) fusion proteins in BL21 codon plus *E. coli* cells (Stratagene). Kinase inactive GST-MPKs (1 μg) were incubated in 20 μl of kinase reaction buffer (50 mM Tris [pH 7.5], 1 mM DTT, 10 mM MgCl$_2$, 0.1 mM ATP, and 6 μCi of ^{32}P-ATP) with immunoprecipitated HA epitope-tagged MKK2 from protoplasts, either without or with previous activation of MKK2 by cold treatment (10 min at 0°C) or of MKK1 by H$_2$O$_2$ treatment (2 mM, 10 min). Kinase inactive GST-MPK fusion proteins were generated by exchanging a conserved lysine residue in the ATP binding domains to methionine and arginine using the Quik-Change kit from Stratagene. The point mutations were K66M, K67R for MPK3; K72M, K73R for MPK4; and K92M, K93R for MPK6, respectively. Kinase reactions were stopped after 30 min by adding 4 μl SDS loading buffer and heating for 5 min at 95°C. Reaction products were analyzed by SDS-PAGE, autoradiography, and Coomassie brilliant blue R250 staining.

Transient Expression Assays
The open reading frames of MPK1 to MPK7 and of MKK1 and MKK2 were cloned into the plant expression vector pRT100 (Töpfer et al., 1987, Kiegerl et al., 2000) and fused at their C-terminal end either to a triple hemagglutinin (HA) epitope (MPKs) or to a c-MYC epitope (MKKs). *Arabidopsis* protoplasts transient expression assays were done as described (Ouaked et al., 2003).

Immunocomplex Kinase Assays
Immunocomplex kinase assays were done according to Cardinale et al. (2002).

Analysis of *MKK2* Null Lines
A MKK2 T-DNA null line (Garlic_511_H01.b.1a.Lb3Fa) was obtained from the Syngenta *Arabidopsis* Insertion Library (SAIL), Torrey Mesa Research Institute (San Diego, CA). Seeds were sown on BASTA-containing media and single BASTA-resistant plants were propagated as individual lines and analyzed by PCR, using gene-specific (MKK2-Nt) and T-DNA-specific (LB1) primers. Insertion of the T-DNA into intron 5 of MKK2 was confirmed by sequencing of the PCR products (Figure 5A). Further selection of the *MKK2* null lines was done by additional PCR analysis using gene specific primers, priming at the N and C terminus of MKK2 (MKK2 Nt and MKK2 Ct) to amplify the genomic MKK2 fragment (1.9 kb for the wild-type *MKK2* gene and 6.7 kb with the T-DNA insertion). Lines putatively homozygous for the T-DNA insertion were subjected to RT-PCR analysis and Southern blotting.

Generation of MKK2-Overexpressing Plants
Wild-type (WT) or constitutive active MKK2 (EE) was cloned into the binary plant expression vector pGreenII 0029 (Hellens et al., 2000) under control of the 35S promoter and transformed as MYC-epitope-tagged versions using the floral dipping method (Clough and Bent, 1998) into Col-0 wild-type plants. Transformed plants were selected by growth on kanamycin-containing media. Plants of the second generation after transformation were used for the experiments.

Cold and Salt Stress Treatments of Plants
Plants sown on agar plates were transferred to soil after 10 days and grown for 3 weeks under long day conditions (16 hr light). Subsequently, one set of plants was incubated for 30 hr at −4°C in the dark (cold shock). Another set of plants was incubated for 24 hr at +4°C (acclimation period) and then subjected to the freezing treatment. A third set of plants was not treated at all and used as control group. To minimize light stress effects, plants were kept out of direct light in the growth chamber for 24 hr after the freezing treatment before returning them to normal light conditions. Pictures were taken 3 days after freezing treatment. Effects of salt on the germination ability were examined by sowing sterilized seeds on 0.5× MS plates with different salt concentrations (0, 100, and 150 mM NaCl, respectively). A total number of about 150 plants were counted per salt concentration.

RNA Isolation from *Arabidopsis* Leaves
Leaves from 2-week-old *Arabidopsis* plants were detached and immediately frozen in liquid nitrogen. One hundred milligrams of leaf material was processed in one sample. RNA was isolated according to manufacturer's instruction using RNeasy plant mini kit (Qiagen, Hilden, Germany). Concentration and purity of RNA was determined by measuring OD at 260 nm and 280 nm.

RT-PCR
RT-PCR was carried out according to Romeis et al. (2001). PCR products were separated on 1.5% agarose gels. The following primers were used. At4g11280: ACS6-1, 5′-CCACCCTGTCATTGTAAG-3′, and ACS6-2, 5′-GAGCGGCGGCGCAACCGGAG-3′. At2g37620: ACT3-1, 5′-ATGGTTAAGGCTGGTTTTGC-3′, and ACT3-2, 5′-AGC ACAATACCGGTAGTACG-3′. At4g25470: CBF2-1, 5′-TCTGAAATG TTTGGCTCCG-3′ and CBF2-2, 5′-CTTCATCCATATAAAACGC-3′. At5g05580: FAD8-1, 5′-GCCATGACTGCGGACATGG-3′, and FAD8-2, 5′-AGATGATAATGTGGGATCTGCGG-3′. At4g29810: MKK2-1, 5′-CCA AATTCCTGACTCAAAGCGG-3′, and MKK2-2, 5′-CTTCTCCTCTGTGG TTGATC-3′. At4g01370: MPK4-1, 5′-GGAATTGTCTGTGCTGCTACA-3′, and MPK4-2, 5′-CCAGCAAATCGACTGCACCAG-3′. At2g39800: P5CS-1, 5′-GGAGGAGCTAGATCGTTCAC-3′, and P5CS-2, 5′-TCAG TTCCAACGCCAGTAGA-3′. At1g13620: RAV1-1, 5′-ATGGAATCGA GTAGCGTTGA-3′, and RAV1-2, 5′-CCGACGTTAACAACGTAA-3′. At1g27730: STZ-1, 5′-ATGGCGCTCGAGGCTCTTAC-3′, and STZ-2, 5′-TCCTTCGTAGTGGCACCGC-3′.

Transcriptome Analysis of MKK2-Overexpressing Lines
Total RNA was prepared from MKK2-WT4 and MKK2-EE4-overexpressing lines and from Col-0 wild-type plants. Transcriptome analysis was performed using the GeneChip *Arabidopsis* ATH1 Genome Array from Affymetrix (Santa Clara, CA). This array contains probe sets representing approximately 24,000 *Arabidopsis* genes. Processing of the RNA samples, hybridization, washing, and scanning of the arrays as well as basic analysis was performed as described in Carson et al. (2002). Signal intensities were normalized by setting target gene intensities (average signal intensity of chip) to 625. Expression signals lower than 125 (25% of target intensity) were floored to 125 to eliminate noise and the number of minor significant expression changes (Zhu and Wang, 2000). Expression ratios were calculated comparing the signal intensities of the two analyzed lines to those of wild-type plants. The resulting data matrix was loaded in Cluster (Eisen et al., 1998). Genes showing at least a 3-fold expression difference in one of the two expression ratios (MKK2-WT4/ wild-type or MKK2-EE4/wild-type) were included in the final data set and subjected to hierarchical clustering as described in Maleck et al. (2000). The final clustergram was displayed using TreeView (Eisen et al., 1998). Functional classification of the genes was done

according to their annotation either in the MIPS (http://mips.gsf.de/proj/thal/db/index.html) or the TAIR database (http://www.Arabidopsis.org/).

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