

A Major Role of the MEKK1–MKK1/2–MPK4 Pathway in ROS Signalling

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ABSTRACT Over the last few years, it has become evident that reactive oxygen species (ROS) signalling plays an important role in various physiological responses, including pathogen defense and stomatal opening/closure. On the other hand, ROS overproduction is detrimental for proper plant growth and development, indicating that the regulation of an appropriate redox balance is essential for plants. ROS homeostasis in plants involves the mitogen-activated protein kinase (MAPK) pathway consisting of the MAPK kinase kinase MEKK1 and the MAPK MPK4. Phenotypic and molecular analysis revealed that the MAPK kinases MKK1 and MKK2 are part of a cascade, regulating ROS and salicylic acid (SA) accumulation. Gene expression analysis shows that of 32 transcription factors reported to be highly responsive to multiple ROS-inducing conditions, 20 are regulated by the MEKK1, predominantly via the MEKK1–MKK1/2–MPK4 pathway. However, MEKK1 also functions on other as yet unknown pathways and part of the MEKK1-dependent MPK4 responses are regulated independently of MKK1 and MKK2. Overall, this analysis emphasizes the central role of this MAPK cascade in oxidative stress signalling, but also indicates the high level of complexity revealed by this signalling network

Key words: Mitogen-activated protein kinases; MEKK1; MKK1; MKK2; MPK4; reactive oxygen species; redox homeostasis; stress signalling; differential gene expression.

INTRODUCTION

Mitogen-activated protein kinase (MAPK) cascades are modules minimally consisting of a MAPK kinase kinase (MAPKKK/MEKK), a MAPK kinase (MAPKK/MKK), and MAPK. Upon activation of the MAPKKK, the signal is transduced via phosphorylation-mediated activation of the downstream MAPKK, which, in turn, phosphorylates and thereby activates its downstream MAPK. The *Arabidopsis* genome contains more than 60 MAPKKKs, 20 MAPKs, and 10 MAPKs, which can combine—depending on the environmental stimulus or developmental stage—into different MAPK modules (MAPK Group, 2002; Nakagami et al., 2005). MAPK cascades are key players in ROS signalling (Nakagami et al., 2005; Pitzschke and Hirt, 2006). Several studies have shown that ROS are not only the trigger, but also the consequence of activation of MAPK signalling pathways (Kovtun et al., 2000; Ren et al., 2002; Yoshioka et al., 2003; Rentel et al., 2004; Nakagami et al., 2004).

For about two million years, molecular oxygen arising from photosynthetic processes has become pivotal to almost all organisms. Reactive oxygen species (ROS), the partially reduced or activated derivatives of oxygen (H_2O_2 , HO^\bullet , $^1\text{O}_2$, O_2^-) are the highly reactive by-products of aerobic metabolism. A sophisticated ROS network comprising antioxidative

enzymes, antioxidants, and ROS-producing enzymes allows a plant to tightly control ROS levels. ROS, arising as toxic by-products of various chemical reactions, can lead to oxidative damage and destruction of cells. For this purpose, plants have developed efficient strategies for targeted production of ROS as well as sophisticated scavenging mechanisms for regulating ROS levels (Hoerberichts and Woltering, 2003; Mittler et al., 2004; Pitzschke and Hirt, 2006). The characterization of the MAPKKK MEKK1 as a regulator of redox homeostasis and its role as upstream regulator of the MAPKKs MKK1 and MKK2 and the downstream MAPK MPK4 opened new possibilities to understand the role of this MAPK cascade in ROS signalling. In this work, we present the molecular analysis of this pathway, with a particular focus on its function in ROS-regulated gene expression.

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RESULTS

ROS-Dependent Gene Regulation

Previous studies have indicated that, depending on the type of ROS (hydrogen peroxide, superoxide, or singlet oxygen) or its sub-cellular production site (plastidic, cytosolic, peroxisomal, or apoplasmic), a different physiological, biochemical, and molecular response is provoked. The specificity of ROS-driven transcript expression had been assessed in a recent study comparing transcriptome data generated from ROS-related microarray experiments (Gadjev et al., 2006). A comparison of datasets obtained by exogenous application of oxidative stress-causing agents (methyl viologen, *Alternaria alternata* toxin, 3-aminotriazole, and ozone) and from the fluorescent (*flu*) mutant and transgenic plants, in which the activity of individual antioxidant enzymes (catalase, cytosolic ascorbate peroxidase, and copper/zinc superoxide dismutase) was perturbed, led to the identification of marker transcripts specifically regulated by hydrogen peroxide, superoxide, or singlet oxygen. Also, several transcripts have been identified as general oxidative stress response markers based on their responsiveness to several of the ROS-generating stresses.

The MEKK1–MKK1/2–MPK4 Pathway Is a Central Regulator of ROS Metabolism

A wide range of environmental stimuli, including bacterial and fungal elicitors as well as diverse abiotic stresses, can initiate MAPK cascades. They can be perceived by as yet mostly unknown receptors that then transduce the signal to the MAPK cascade. However, secondary signals that are produced by the challenged plant can be involved also. Examples are the plant-derived peptide systemin, which is formed upon wounding (McGurl et al., 1992), and the plant hormone salicylic acid (SA), which is synthesised in a stress-dependent manner and essential for many biotic stress responses (Koorneef and Pieterse, 2008). As previous studies have shown, stress, SA, ROS, and MAPK cascades are strongly interconnected (Nakagami et al., 2005; Pitzschke and Hirt, 2006).

MEKK1 was one of the first MAPKKs to be characterized in *Arabidopsis*; the gene is transcriptionally up-regulated in response to touch, cold, and salt stress (Mizoguchi et al., 1996; Covic et al., 1999). MEKK1 has been implicated in the biotic and abiotic activation of the MAPKs MPK3, MPK4, and MPK6 (Asai et al., 2002; Teige et al., 2004). Moreover, MEKK1 can be activated by H₂O₂ through post-translational modifications including proteasome-mediated MEKK1 protein stabilization. *Mekk1* homozygous knockout plants show a severe dwarfism, accumulate high amounts of ROS, and develop local lesions reminiscent of programmed cell death (PCD) (Ichimura et al., 2006; Nakagami et al., 2006). Transcription of a number of genes encoding redox-regulatory enzymes is strongly mis-expressed in *mekk1* knockout plants (Ichimura et al., 2006; Nakagami et al., 2006). Whereas homozygous *mpk4* mutants display a similar lethal phenotype as *mekk1* mutants, no such

effects are observed in *mpk3* or *mpk6* mutant plants. Similarly, altered expression of redox-regulatory genes and the age-dependent lethal phenotype were only found in *mpk4*, but not in *mpk3* mutants (Nakagami et al., 2006).

Because MEKK1 is required for H₂O₂-induced activation of MPK4, but not of MPK3 or MPK6, a major role in ROS homeostasis was ascribed to MEKK1 and its downstream target MPK4. Programmed cell death often is associated with the concurrent accumulation of ROS and salicylic acid (SA). Indeed, *mekk1* and *mpk4* mutants were found to contain highly elevated SA levels and to constitutively express SA-dependent stress genes (Ichimura et al., 2006; Nakagami et al., 2006). The dwarfed phenotype of *mekk1* and *mpk4* plants can be partially rescued by the expression of the bacterial SA-degrading *NahG* enzyme (Petersen et al., 2000; Suarez-Rodriguez et al., 2007). These data suggest that the mutant phenotypes of *mekk1* and *mpk4* plants are predominantly due to elevated SA levels. However, SA and ROS do not always seem to go together with cell death. The activation-tagged *bud1* mutant plants, which overexpress the MAPKK *MKK7*, have elevated SA levels and exhibit constitutive pathogenesis-related (*PR*) gene expression but no spontaneous lesions (Zhang et al., 2007), indicating that ROS generation can be uncoupled from SA signalling.

Interestingly, expression of a kinase-impaired version of *MEKK1(K361M)* also largely rescued the dwarfism, *PR* gene expression, and the callose deposition phenotype of *mekk1* mutant plants (Suarez-Rodriguez et al., 2007). *mekk1* mutant plants are impaired in flagellin (*flg22*)-mediated activation of MPK4, but not of MPK3 or MPK6 and ectopic expression of *MEKK1(K361M)* leads to wild-type profiles of MAPK activation in the *mekk1* mutants. Although it cannot be excluded that residual kinase activity may reside in the MEKK1(K361M) protein, it appears that the kinase activity of MEKK1 may be dispensable for at least some of its in-planta functions (Suarez-Rodriguez et al., 2007).

ROS-Related Transcriptome Analysis of *mekk1*, *mkk1/2*, and *mpk4* Mutants

Previous studies have shown MEKK1 to be an activator of two highly homologous MAPKKs (MKK1 and MKK2), which function upstream of the MAPKs MPK4 and MPK6 (Teige et al., 2004). All of these components have been implicated in biotic, abiotic, and ROS signalling (Nakagami et al., 2005). Moreover, *Arabidopsis* mutants lacking *mekk1*, *mkk1/2*, or *mpk4* show striking similarities in many aspects, including a dwarfed phenotype, formation of spontaneous lesions as well as accumulation of ROS and SA.

In order to gain a global view on the molecular processes affected in these mutants, we performed a full genome transcriptome analysis. In agreement with the results from Qiu et al. (2008), a strong overlap in the transcriptome patterns of *mekk1*, *mkk1/2*, and *mpk4* plants confirmed that MEKK1, MKK1, MKK2, and MPK4 are components of the same MAPK module (Figures 1 and 2).

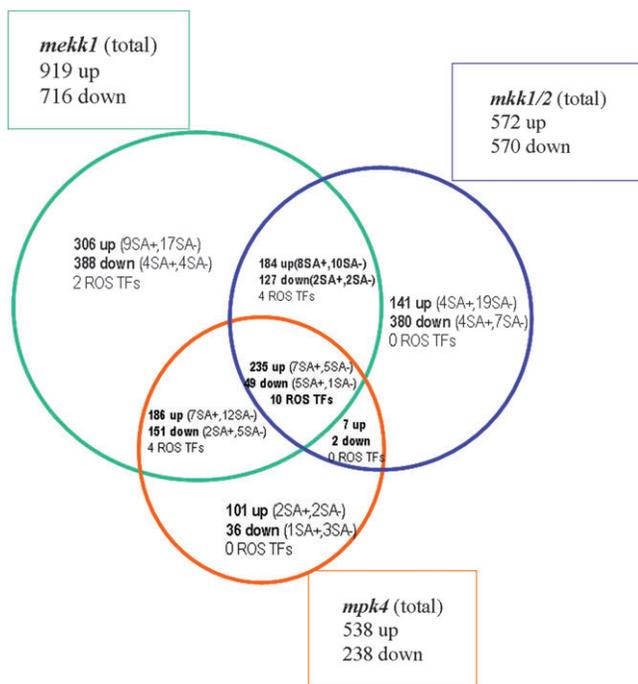


Figure 1. Overlap of Transcriptome Patterns of *mekk1*, *mpk4*, and *mkk1/2* Mutants.

Within a given dataset, the number of genes with reported induction (SA+) or repression (SA-) by salicylic acid (Schenk et al., 2000) as well as the distribution of general ROS-responsive transcription factors (TF) (Gadjjev et al., 2006) are shown. See text for details.

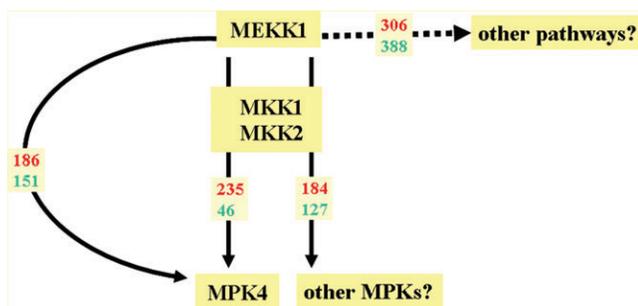


Figure 2. Regulation of Gene Expression by MEKK1 via MKK1/MKK2 and MPK4-Dependent and -Independent Pathways.

The numbers of genes (red: up-; green: down-regulated in the respective mutants) controlled via the individual pathways are indicated.

Regulation of Common Stress-Responsive Genes by MEKK1–MKK1/2–MPK4

Recently, a genome-wide bioinformatical analysis of microarray data has established a list of 197 genes with responsiveness to cold, osmotic stress, wounding as well as to biotic stress (Ma and Bohnert, 2007). These common stress-induced genes also include genes encoding MAPK components: *MPK5*, *MKK9*, and *MAPKKK14*. Also *ACS6*, encoding the rate-limiting enzyme of

ethylene biosynthesis and a substrate for MPK6 (Liu and Zhang, 2004) as well as six ERF/AP2 transcription factors (*AtERF*) were among the common stress genes. Therefore, ethylene signalling-mediated engagement of a subset of the MAPK family as a component of the common stress response has been proposed (Ma and Bohnert, 2007).

Of the 197 common stress-responsive genes reported by Ma and Bohnert (2007), 54 are differentially expressed (all up-regulated!) in *mekk1*, *mkk1/mkk2*, and/or *mpk4* knockout plants (Table 1). Transcription factor-encoding genes are clearly overrepresented. Almost all of those common stress genes with enhanced transcript levels in *mpk4* and/or *mkk1/2* are also up-regulated in *mekk1*: 19 genes are common to all three mutants, 10 are specific to *mekk1* and *mkk1/2*, 34 are specific to *mekk1* and *mpk4* mutants. These findings strongly suggest that in the context of stress signalling, the signal mediated to MKK1/2 and/or MPK4 almost exclusively has been transduced via MEKK1.

SA-Dependent Gene Expression in *mekk1*, *mkk1/2*, and *mpk4* Mutants

The accumulation of SA and H₂O₂ in *mekk1*, *mkk1/2*, and *mpk4* mutants is reflected by the strong overlap of genes differentially expressed in these mutants with known SA- or ROS-dependent genes. Of 441 and 254 SA-regulated genes (at least two-fold up or down, respectively) (Schenk et al., 2000), 142 have altered transcript levels in at least one of the mutants *mekk1*, *mpk4*, and/or *mkk1/2*. Interestingly, these genes represent all possible combinations with respect to enhanced or repressed expression in the mutants versus SA response (up/up, up/down, down/up, and down/down) (Figure 1 and Table 2). These findings are indicative of an expression dynamics in a number of SA-regulated genes: short-term exposure (by exogenous treatment for 24 h; Schenk et al., 2000) results in enhanced expression, whereas long-term exposure (as in SA-accumulating mutants) leads to decreased expression, or vice versa. Thus, in the SA-accumulating mutants, negative feedback mechanisms counteracting the effects of constantly high SA levels might have been initiated over the prolonged period. Good candidates involved in this feedback regulation might be ROS: while short-term SA leads to an oxidative burst and induction of genes encoding products that further accelerate the SA-triggered processes, proteins arising from constitutively high SA-mediated gene induction might have accumulated to amounts exceeding a certain threshold, which then act as negative regulators of other SA-responsive genes. This feedback regulation might help the plant to prevent excessive induction of stress genes whose products strongly interfere with proper development, namely leading to even more severe phenotypes than those observed for *mekk1*, *mkk1/2*, and *mpk4* mutants.

ROS-Responsive Gene Expression in *mekk1*, *mkk1/2*, and *mpk4* Mutants

A comparison of the *mekk1*, *mkk1/2*, and *mpk4* transcriptome with genes related to oxidative stress revealed a strong

Table 1. List of Genes Differentially Expressed in *mekk1*, *mkk1/2*, and/or *mpk4* Overlapping with the Dataset of 197 Genes Responsive to Multiple Stresses (Ma and Bohnert, 2007).

gene ID	function	mekk1	mpk4	mkk1/2	ROS-responsive
AT5G47120	ATBI-1 (ARABIDOPSIS BAX INHIBITOR 1)	up	up	up	
AT3G50930	ATP binding / ATPase	up	up	up	
AT2G41410	calcium ion binding_ calmodulin, putative	up	up	up	
AT2G46600	calcium ion binding	up	up	up	
AT3G10300	calcium ion binding	up	up	up	ROS
AT5G26340	MSS1; carbohydrate transporter/ sugar porter_ hexose transporter, putative	up	up	up	
AT3G60420	unknown protein	up	up	up	
AT5G20230	ATBCB (ARABIDOPSIS BLUE-COPPER-BINDING PROTEIN)	up	up	up	
AT1G19020	unknown protein	up	up	up	ROS
AT5G35735	dopamine beta-monooxygenase_ auxin-responsive family protein	up	up	up	
AT1G09070	rhodopsin-like receptor_ C2 domain-containing protein / src2-like protein, putative	up	up	up	
AT1G80840	WRKY40	up	up	up	ROS
AT2G30250	WRKY25	up	up	up	ROS
AT2G40140	transcription factor_ zinc finger (CCCH-type) family protein	up	up	up	ROS
AT3G55980	transcription factor_ zinc finger (CCCH-type) family protein	up	up	up	ROS
AT4G31800	WRKY18	up	up	up	
AT4G14365	ubiquitin-protein ligase/ zinc ion binding_ zinc finger (C3HC4-type RING finger)	up	up	up	
AT5G59820	RHL41 (RESPONSIVE TO HIGH LIGHT 41) zinc finger (C2H2 type) family protein (ZAT12)	up	up	up	ROS
AT2G38470	WRKY33	up	up	up	ROS
AT2G46620	ATP binding / ATPase	up	up		ROS
AT2G22500	binding / transporter_ mitochondrial substrate carrier family protein	up	up		ROS
AT3G52800	DNA binding / zinc ion binding_ zinc finger (AN1-like) family protein	up	up		
AT1G73500	ATMKK9; kinase	up	up		ROS
AT1G25400	unknown protein	up	up		
AT1G32920	unknown protein	up	up		ROS
AT2G27830	unknown protein	up	up		ROS
AT2G36220	unknown protein	up	up		
AT3G10930	unknown protein	up	up		ROS
AT1G25560	AP2 domain-containing transcription factor, putative	up	up		ROS
AT3G50260	AP2 domain-containing transcription factor, putative	up	up		ROS
AT1G28370	transcriptional repressor_ ERF domain protein 11 (ERF11)	up	up		ROS
AT1G27730	zinc ion binding_ zinc finger (C2H2 type) family protein (ZAT10)	up	up		ROS
AT3G16720	ATL2_ zinc finger (C3HC4-type RING finger) family protein	up	up		ROS
AT3G46620	ubiquitin-protein ligase/ zinc ion binding_ zinc finger (C3HC4-type RING finger) family protein	up	up		ROS
AT5G17760	ATP binding	up		up	
AT4G27940	mitochondrial substrate carrier family protein	up		up	ROS
AT4G18010	IP5PII (INOSITOL POLYPHOSPHATE 5-PHOSPHATASE II)	up		up	ROS
AT1G76360	protein kinase	up		up	
AT1G19180	unknown protein	up		up	ROS
AT2G22860	ATPSK2 (PHYTOSULFOKINE 2 PRECURSOR)	up		up	
AT5G42050	unknown protein	up		up	
AT3G62260	protein phosphatase type 2C	up		up	

Table 1. Continued

gene ID	function	mekk1	mpk4	mkk1/2	ROS-responsive
AT3G49530	ANAC062; transcription factor_ no apical meristem (NAM) family protein	up		up	
AT4G17230	transcription factor_ scarecrow-like transcription factor 13 (SCL13)	up		up	ROS
AT1G19380	unknown protein	up			
AT1G27100	unknown protein	up			
AT1G61340	unknown protein_ F-box family protein	up			ROS
AT3G02150	PTF1 (PLASTID TRANSCRIPTION FACTOR 1)	up			
AT1G02660	triacylglycerol lipase_ lipase class 3 family protein	up			ROS
AT3G19580	AZF2 (ARABIDOPSIS ZINC-FINGER PROTEIN 2) transcription factor	up			
AT1G58420	unknown protein		up		ROS
AT5G49280	unknown protein_ hydroxyproline-rich glycoprotein family protein			down	
AT5G62570	calmodulin binding			up	
AT1G74450	unknown protein			up	ROS

Many of these genes are responsive to ROS (right column). Genes encoding transcription factors are highlighted.

overlap. In the study of Gadjev et al. (2006), several transcription factor genes responsive to multiple oxidative stresses had been identified. These transcription factors are candidates that could be responsible for orchestrating the specific transcriptional signatures triggered by different ROS. Using the ROS gene dataset (Gadjev et al., 2006), we investigated the abundance of genes responsive to individual or multiple ROS-generating stresses in the datasets of genes differentially expressed in the *mekk1*, *mkk1/2*, and *mpk4* mutants (Tables 3A–3C).

Of 701 genes specifically responsive to hydrogen peroxide (193), superoxide (194), or singlet oxygen (314), 120 have altered expression in at least one of the mutants *mekk1*, *mkk1/2*, and *mpk4*; and many are shared by all mutants. There is no apparent preference for hydrogen peroxide-, superoxide- or singlet oxygen-responsive genes in any of the mutants' differential transcriptomes, indicating major disturbances in the balances of all ROS species in these mutants. This is in line with the finding that a large number of ROS scavenging enzyme-encoding genes have altered transcript levels in the mutants (see below). Of 103 genes commonly responsive to various types of ROS, 51 are differentially expressed in *mekk1*, *mkk1/2*, and/or *mpk4*. Among the 32 transcription factors showing a very strong expression response to oxidative stress in general (Gadjev et al., 2006), as many as 20 are differentially expressed (all up-regulated) in *mekk1*, *mkk1/2*, and/or *mpk4* mutant plants (Tables 3A and 3C, and Figure 1). Whereas none of these 20 transcription factor genes is exclusively affected in *mpk4* or *mkk1/2*, two are specific for *mekk1*, four are shared by *mkk1/2* and *mekk1*, four are shared by *mpk4* and *mekk1*, and 10 are common to all three mutants. These findings strongly support a role of MEKK1 as a major regulator of redox homeostasis, which is mainly controlled through the MEKK1–MKK1/2–MPK4 cascade.

ROS Scavenging Mechanisms Are Disturbed in *mekk1*, *mkk1/2*, and *mpk4* Mutants

Redox homeostasis is achieved through the action of ROS scavenging enzymes. In *Arabidopsis*, ROS scavenging enzymes are encoded by 148 genes representing 15 gene families (Mittler et al., 2004). Not unexpectedly, a significant proportion of these genes, 23, representing nine of the 15 gene families, are differentially expressed in *mekk1*, *mkk1/2*, and/or *mpk4* plants, indicating major imbalances in redox homeostasis (Table 4). The corresponding gene products are of diverse sub-cellular localization, indicating that redox imbalances due to loss of MEKK1, MKK1/MKK2, and/or MPK4 activity are not restricted to a particular cell compartment. For instance, all three mutant lines have reduced transcript levels of the gene encoding thylakoidal peroxidase (*tAPX*), which uses ascorbic acid for peroxide removal ($2\text{Asc} + \text{H}_2\text{O}_2 \rightarrow 2\text{MDA} + 2\text{H}_2\text{O}$) (Table 4). The repression of *tAPX* in *mekk1*, *mkk1/2*, and *mpk4* is likely to contribute to the enhanced ROS levels in these mutants. The importance of *tAPX* in the removal of H_2O_2 has been emphasized in previous studies showing that overexpression of *tAPX* renders plants more tolerant to treatment with the superoxide-generating herbicide paraquat (PQ) (Murgia et al., 2004), whereas antisense reduction of *tAPX* results in PQ hypersensitivity (Tarantino et al., 2005). In addition to *tAPX*, the expression of all three members of the *CAT* family, encoding the peroxisomal hydrogen peroxide-scavenging enzyme catalase (*CAT*) ($2\text{H}_2\text{O}_2 \rightarrow \text{H}_2\text{O} + \text{O}_2$) is affected in our analyzed mutants. Interestingly, there are differences between the mutants with respect to differential expression of individual catalase genes. The expression of *CAT2* encoding the major H_2O_2 scavenging enzyme (Vandenabeele et al., 2004) is reduced in *mekk1* and *mpk4*, but unaffected in *mkk1/2*. This repression is likely to contribute to the highly elevated levels of

Table 2. List of SA-Responsive Genes that Are Differentially Expressed in *mekk1*, *mkk1/2*, and/or *mpk4* Mutants.

gene ID	function	mekk1	mpk4	mkk1/2	SA responsive
AT1G03870	FLA9 (fasciclin-like arabinogalactan-protein 9)	down	down	down	SA+
AT2G10940	lipid transfer protein (LTP) family protein	down	down	down	SA+
AT4G14040	selenium binding	down	down	down	SA+
AT5G13930	CHS (CHALCONE SYNTHASE)	down	down	down	SA+
AT5G44020	acid phosphatase class B family protein	down	down	down	SA+
AT4G39330	oxidoreductase/ zinc ion binding unknown protein	down	down	down	SA-
AT1G04680	lyase/ pectate lyase	down	down		SA+
AT1G12110	NRT1.1; transporter	down	down		SA+
AT1G32060	uridine kinase_ phosphoribulokinase (PRK) / phosphopentokinase	down	down		SA+
AT1G68560	XYL1; hydrolase	down	down		SA+
AT2G28000	CPN60A RuBisCO subunit binding-protein alpha subunit	down	down		SA+
AT2G29630	thiamine biosynthesis family protein	down	down		SA+
AT2G43710	SSI2; acyl-[acyl-carrier protein] desaturase	down	down		SA+
AT3G01500	CA1 (CARBONIC ANHYDRASE 1); carbonate dehydratase	down	down		SA+
AT3G28040	leucine-rich repeat transmembrane protein kinase, putative	down	down		SA+
AT3G48560	CSR1 (CHLORSULFURON/IMIDAZOLINONE RESISTANT 1)	down	down		SA+
AT4G35090	CAT2 (CATALASE 2)	down	down		SA+
AT5G09870	CESA5 (CELLULOSE SYNTHASE 5)	down	down		SA+
AT1G09780	2,3-bisphosphoglycerate-independent phosphoglycerate mutase	down	down		SA-
AT1G75280	unknown protein_ isoflavone reductase, putative	down	down		SA-
AT2G32990	glycosyl hydrolase family 9 protein	down	down		SA-
AT2G41820	leucine-rich repeat transmembrane protein kinase, putative	down	down		SA-
AT3G23820	GAE6 (UDP-D-GLUCURONATE 4-EPIMERASE 6)	down	down		SA-
AT1G74670	unknown protein_ gibberellin-responsive protein, putative	down		down	SA+
AT2G05990	MOD1 (MOSAIC DEATH 1); enoyl-[acyl-carrier protein] reductase	down		down	SA+
AT2G18230	inorganic diphosphatase/ magnesium ion binding	down		down	SA+
AT2G34430	LHB1B1 chlorophyll A-B binding protein / LHClI type I (LHB1B1)	down		down	SA+
AT2G36830	GAMMA-TIP; water channel_ major intrinsic family protein	down		down	SA+
AT3G07010	lyase/ pectate lyase	down		down	SA+
AT4G17340	DELTA-TIP2/TIP2;2; water channel_ major intrinsic family protein	down		down	SA+
AT5G09220	AAP2 (AMINO ACID PERMEASE 2)	down		down	SA+
AT5G20630	GLP3 (GERMIN-LIKE PROTEIN 3); nutrient reservoir	down		down	SA+
AT5G45670	GDSL-motif lipase/hydrolase family protein	down		down	SA+
AT2G04780	FLA7 (fasciclin-like arabinogalactan-protein 7))	down		down	SA-
AT3G55120	TT5 (TRANSPARENT TESTA 5); chalcone isomerase	down		down	SA-
AT1G72610	GLP1 (GERMIN-LIKE PROTEIN 1); nutrient reservoir	down			SA+
AT1G74470	geranylgeranyl reductase	down			SA+
AT1G75680	glycosyl hydrolase family 9 protein	down			SA+
AT1G75820	CLAVATA1 receptor kinase (CLV1)	down			SA+
AT2G21330	fructose-bisphosphate aldolase	down			SA+
AT2G33800	ribosomal protein S5 family protein	down			SA+
AT2G39730	RCA (RUBISCO ACTIVASE)	down			SA+
AT3G12780	PGK1 (PHOSPHOGLYCERATE KINASE 1)	down			SA+
AT3G14420	glycolate oxidase/ oxidoreductase	down			SA+
AT3G17510	CIPK1 (CBL-INTERACTING PROTEIN KINASE 1)	down			SA+
AT3G44990	XTR8 xyloglucan endotransglycosylase	down			SA+
AT3G51895	SULTR3;1 (SULFATE TRANSPORTER 1)	down			SA+
AT3G54050	fructose-bisphosphatase	down			SA+

Table 2. Continued

gene ID	function	mekk1	mpk4	mkk1/2	SA responsive
AT3G62030	ROC4; peptidyl-prolyl cis-trans isomerase	down			SA+
AT4G21750	ATML1 (MERISTEM LAYER 1); specific homeobox gene	down			SA+
AT4G24770	RBP31 (31-KDA RNA BINDING PROTEIN)	down			SA+
AT4G32410	CESA1 (CELLULASE SYNTHASE 1)	down			SA+
AT1G23480	ATCSLA03 glycosyl transferase family 2 protein	down			SA-
AT1G51805	leucine-rich repeat protein kinase, putative	down			SA-
AT2G24230	leucine-rich repeat transmembrane protein kinase, putative	down			SA-
AT3G04870	ZDS (ZETA-CAROTENE DESATURASE)	down			SA-
AT1G31580	ECS1	up	up	up	SA+
AT1G80440	unknown protein_ kelch repeat-containing F-box family protein	up	up	up	SA+
AT2G18700	ATTPS11 glycosyl transferase family 20 protein	up	up	up	SA+
AT4G02380	SAG21 (SENESCENCE-ASSOCIATED GENE 21)	up	up	up	SA+
AT4G02520	ATGSTF2; glutathione transferase	up	up	up	SA+
AT4G31500	CYP83B1 (CYTOCHROME P450 MONOOXYGENASE 83B1)	up	up	up	SA+
AT4G33300	ADR1-L1; disease resistance protein (CC-NBS-LRR class), putative	up	up	up	SA+
AT1G18570	MYB51 transcription factor	up	up	up	SA-
AT3G04720	PR4 (PATHOGENESIS-RELATED 4)_ hevein-like protein (HEL)	up	up	up	SA-
AT3G55980	transcription factor_ zinc finger (CCCH-type) family protein	up	up	up	SA-
AT3G57260	BGL2 (PATHOGENESIS-RELATED PROTEIN 2) glycosyl hydrolase family 17	up	up	up	SA-
AT4G17500	ERF-1 ethylene-responsive element-binding factor 1	up	up	up	SA-
AT1G28330	DRM1 (DORMANCY-ASSOCIATED PROTEIN 1)	up	up		SA+
AT1G32210	ATDAD1 (DEFENDER AGAINST APOPTOTIC DEATH 1)	up	up		SA+
AT1G45145	ATTRX5; thiol-disulfide exchange intermediate_ thioredoxin H-type 5 (TRX-H-5)	up	up		SA+
AT2G05380	GRP3S (GLYCINE-RICH PROTEIN 3 SHORT ISOFORM)	up	up		SA+
AT3G16770	ATEBP/RAP2.3 AP2 domain-containing protein	up	up		SA+
AT4G08150	KNAT1 (BREVIPEDICELLUS 1); transcr factor_ homeobox protein	up	up		SA+
AT4G35770	SEN1 (DARK INDUCIBLE 1)_ senescence-associated protein (SEN1)	up	up		SA+
AT2G37750	unknown protein	up	up		SA-
AT3G28950	unknown protein_ avirulence-responsive protein-related	up	up		SA-
AT1G03090	MCCA unknown protein	up		up	SA+
AT1G20620	CAT3 (CATALASE 3); (SEN2)	up		up	SA+
AT1G80460	NHO1; carbohydrate kinase_ glycerol kinase, putative	up		up	SA+
AT3G03470	CYP89A9; heme binding / iron ion binding / monooxygenase/ oxygen binding	up		up	SA+
AT3G44300	NIT2 (NITRILASE 2)_ nitrilase 2 (NIT2)	up		up	SA+
AT4G37520	peroxidase_ peroxidase 50 (PER50) (P50) (PRXR2)	up		up	SA+
AT5G14780	FDH (FORMATE DEHYDROGENASE); oxidoreductase	up		up	SA+
AT5G39190	GLP2A (GERMIN-LIKE PROTEIN 2A); nutrient reservoir	up		up	SA+
AT4G15530	pyruvate phosphate dikinase	up		up	SA-
AT5G07440	GDH2 (GLUTAMATE DEHYDROGENASE 2)	up		up	SA-
AT1G62300	WRKY6; transcription factor	up			SA+
AT2G05940	protein kinase	up			SA+
AT2G21620	RD2 (responsive to dessication protein)	up			SA+
AT2G33150	PED1 (PEROXISOME DEFECTIVE 1)_ acetyl-CoA C-acyltransferase	up			SA+
AT3G12610	DRT100 (DNA-DAMAGE REPAIR/TOLERATION 100)	up			SA+
AT3G14990	thiazole biosynthesis protein, putative	up			SA+
AT3G21720	catalytic/ isocitrate lyase_ isocitrate lyase, putative	up			SA+
AT3G61890	ATHB-12 HD-ZIP transcription factor	up			SA+
AT5G19120	unknown protein	up			SA+

Table 2. Continued

gene ID	function	mekk1	mpk4	mkk1/2	SA responsive
AT3G02520	GRF7; protein phosphorylated amino acid binding	up			SA-
AT3G43800	ATGSTU27; glutathione transferase	up			SA-
AT5G11920	glycosyl hydrolase family 32 protein	up			SA-
AT5G54960	PDC2 (PYRUVATE DECARBOXYLASE-2)	up			SA-
AT1G23190	phosphotransferases / phosphoglucomutase		down		SA+
AT1G35720	ANNAT1 (ANNEXIN ARABIDOPSIS 1)		down		SA+
AT1G77670	1-aminocyclopropane-1-carboxylate synthase		down		SA-
AT1G77760	NIA1 (NITRATE REDUCTASE 1)_ nitrate reductase 1 (NR1)		down		SA-
AT3G15950	unknown protein_ DNA topoisomerase-related		down		SA-
AT3G03920	RNA binding / rRNA binding_ Gar1 RNA-binding region family protein		up	down	SA+
AT5G57220	CYP81F2; heme binding / iron ion binding / monooxygenase/ oxygen binding		up	up	SA+
AT2G37410	ATTIM17-2 mitochondrial import inner membrane translocase		up		SA+
AT2G38880	HAP3A_ histone-like transcription factor (CBF/NF-Y) family protein		up		SA-
AT1G12080	unknown protein			down	SA+
AT1G20440	COR47_ dehydrin (COR47)			down	SA+
AT1G51830	leucine-rich repeat protein kinase, putative			down	SA+
AT1G68850	peroxidase			down	SA+
AT1G70850	unknown protein_ Bet v I allergen family protein			down	SA+
AT1G78100	unknown protein_ F-box family protein			down	SA+
AT2G27510	ferredoxin, putative			down	SA+
AT2G32720	B5 #4_ cytochrome b5, putative			down	SA+
AT2G38540	LP1; nonspecific lipid transfer protein 1 (LTP1)			down	SA+
AT4G00360	CYP86A2; oxygen binding ANTR2; organic anion transporter			down	SA+
AT4G25050	ACP4_ acyl carrier family protein / ACP family protein			down	SA+
AT4G29905	unknown protein			down	SA+
AT4G35100	PIP3 (PLASMA MEMBRANE INTRINSIC PROTEIN 3)			down	SA+
AT4G39260	ATGRP8 (GLYCINE-RICH PROTEIN 8)			down	SA+
AT5G02450	60S ribosomal protein L36 (RPL36C)			down	SA+
AT5G09530	unknown protein_ hydroxyproline-rich glycoprotein family protein			down	SA+
AT5G15230	GASA4_ (gibberellin-regulated protein 4)			down	SA+
AT5G47450	AtTIP2;3; water channel_ major intrinsic family protein			down	SA+
AT5G65310	ATHB5_HD-ZIP transcription factor 5			down	SA+
AT1G16920	RAB11; GTP binding_ Ras-related GTP-binding protein, putative			down	SA-
AT2G21660	ATGRP7; RNA binding_ glycine-rich RNA-binding protein (GRP7)			down	SA-
AT2G43610	chitin binding / chitinase_ glycoside hydrolase family 19 protein			down	SA-
AT3G24520	AT-HSFC1;heat shock transcription factor			down	SA-
AT3G50970	dehydrin xero2 / low-temperature-induced protein LTI30			down	SA-
AT5G10430	AGP4 (ARABINOGLACTAN-PROTEIN 4)			down	SA-
AT5G48000	CYP708A2			down	SA-
AT1G72150	PATL1 (PATELLIN 1);SEC14 cytosolic factor family protein			up	SA+
AT2G35940	BLH1 (BEL1-like homeodomain 1)_ homeodomain-containing protein			up	SA+
AT4G23850	long-chain acyl-CoA synthetase			up	SA+
AT5G40170	kinase_ disease resistance family protein			up	SA+
AT1G49750	unknown protein_ leucine-rich repeat family protein			up	SA-
AT1G74100	sulfotransferase_ sulfotransferase family protein			up	SA-
AT3G21220	ATMKK5 (MITOGEN-ACTIVATED PROTEIN KINASE KINASE 5)			up	SA-
AT4G24190	SHD (SHEPHERD); clavata formation protein, putative			up	SA-

Table 3A. Common ROS-Responsive Genes that Are Differentially Expressed in *mekk1*, *mkk1/2*, and/or *mpk4* Mutants.

gene ID	function	mekk1	mpk4	mkk1/2	responsive to multiple ROS
AT1G07000	exocyst subunit EXO70 family protein	up	up	up	ROS
AT1G09970	leucine-rich repeat transmembrane protein kinase, putative	up	up	up	ROS
AT1G18570	MYB51	up	up	up	ROS
AT1G19020	unknown protein	up	up	up	ROS
AT1G28480	glutaredoxin family protein	up	up	up	ROS
AT1G72520	lipoxygenase, putative	up	up	up	ROS
AT1G76600	unknown protein	up	up	up	ROS
AT1G80840	WRKY40	up	up	up	ROS
AT2G26530	AR781	up	up	up	ROS
AT2G30250	WRKY25	up	up	up	ROS
AT2G38470	WRKY33	up	up	up	ROS
AT2G40140	transcription factor_ zinc finger (CCCH-type)	up	up	up	ROS
AT2G43510	ATTI1_ trypsin inhibitor, putative	up	up	up	ROS
AT3G11820	SYP121; t-SNARE_ syntaxin 121	up	up	up	ROS
AT3G23250	MYB15	up	up	up	ROS
AT3G48650	unknown protein	up	up	up	ROS
AT3G54150	S-adenosylmethionine-dependent methyltransferase	up	up	up	ROS
AT3G55980	transcription factor_ zinc finger (CCCH-type)	up	up	up	ROS
AT4G17500	ERF-1 ethylene-responsive element-binding factor 1	up	up	up	ROS
AT4G33050	calmodulin binding	up	up	up	ROS
AT4G39670	unknown protein	up	up	up	ROS
AT5G01540	lectin protein kinase, putative	up	up	up	ROS
AT5G13080	WRKY75	up	up	up	ROS
AT5G47220	ERF-2 ethylene-responsive element-binding factor 2	up	up	up	ROS
AT5G59820	ZAT12	up	up	up	ROS
AT1G25560	AP2 domain-containing transcription factor, putative	up	up		ROS
AT1G27730	ZAT10	up	up		ROS
AT1G32920	unknown protein	up	up		ROS
AT1G73500	ATMKK9; kinase	up	up		ROS
AT2G22500	mitochondrial substrate carrier family protein	up	up		ROS
AT2G43000	ANAC042; transcription factor_ no apical meristem (NAM) family	up	up		ROS
AT3G10930	unknown protein	up	up		ROS
AT3G50260	AP2 domain-containing transcription factor, putative	up	up		ROS
AT1G19180	unknown protein	up		up	ROS
AT1G52890	ANAC019 no apical meristem (NAM) family protein	up		up	ROS
AT1G76590	binding_ zinc-binding family protein	up		up	ROS
AT2G43820	UDP-glycosyltransferase/ transferase	up		up	ROS
AT3G04070	ANAC047 no apical meristem (NAM) family protein	up		up	ROS
AT3G15500	ATNAC3 no apical meristem (NAM) family protein (NAC3)	up		up	ROS
AT4G17230	scarecrow-like transcription factor 13 (SCL13)	up		up	ROS
AT4G18010	IP5PII (INOSITOL POLYPHOSPHATE 5-PHOSPHATASE II)	up		up	ROS
AT1G43160	RAP2.6 AP2 domain-containing protein	up			ROS
AT1G61340	unknown protein_ F-box family protein	up			ROS
AT1G62300	WRKY6	up			ROS
AT2G41380	unknown protein_ embryo-abundant protein-related	up			ROS
AT3G08720	ATPK19 serine/threonine protein kinase	up			ROS
AT3G08970	DNAJ heat shock N-terminal domain-containing protein	up			ROS
AT3G11340	UDP-glycosyltransferase	up			ROS

Table 3A. Continued

gene ID	function	mekk1	mpk4	mkk1/2	responsive to multiple ROS
AT4G23210	kinase	up			ROS
AT2G37940	unknown protein		up		ROS
AT4G23190	CRK11 (CYSTEINE-RICH RLK11); kinase		up		ROS

Transcription factor-encoding genes are highlighted.

Table 3B. Transcripts Specifically Responsive to Singlet Oxygen, Superoxide or H₂O₂ and their Altered Abundance in *mekk1*, *mkk1/2*, and/or *mpk4* Mutants.

gene ID	function	mekk1	mpk4	mkk1/2	singlet oxygen	superoxide	H ₂ O ₂
AT1G64780	ATAMT1;2; ammonium transporter	down	down	down		O ₂ ⁻	
AT1G13650	unknown protein	down	down				H ₂ O ₂
AT1G17050	dimethylallyltranstransferase	down	down			O ₂ ⁻	
AT4G35090	CAT2 (CATALASE 2)	down	down				H ₂ O ₂
AT1G30530	UDP-glycosyltransferase	down		down			H ₂ O ₂
AT1G62250	unknown protein	down		down		O ₂ ⁻	
AT1G73120	unknown protein	down		down		O ₂ ⁻	
AT2G33850	unknown protein	down		down		O ₂ ⁻	
AT4G02290	glycosyl hydrolase family 9 protein	down		down		O ₂ ⁻	
AT4G03060	AOP2 (ALKENYL HYDROXALKYL PRODUCING 2); oxidoreductase	down		down			H ₂ O ₂
AT5G33370	carboxylic ester hydrolase	down		down		O ₂ ⁻	
AT1G01060	LHY (LATE ELONGATED HYPOCOTYL);myb family transcr factor	down				O ₂ ⁻	
AT1G62510	lipid transfer protein (LTP) family protein	down					H ₂ O ₂
AT1G74160	unknown protein	down			¹ O ₂		
AT2G21140	ATPRP2 (PROLINE-RICH PROTEIN 2)	down				O ₂ ⁻	
AT4G23290	kinase	down					H ₂ O ₂
AT4G32770	VTE1 (VITAMIN E DEFICIENT 1)_ tocopherol cyclase	down				O ₂ ⁻	
AT5G04530	beta-ketoacyl-CoA synthase family protein	down			¹ O ₂		
AT1G31580	ECS1	up	up	up			H ₂ O ₂
AT1G69490	NAP (NAC-LIKE, ACTIVATED BY AP3/PI); transcription factor	up	up	up			H ₂ O ₂
AT2G29120	ATGLR2.7_ glutamate receptor family protein	up	up	up			H ₂ O ₂
AT2G44180	MAP2A (METHIONINE AMINOPEPTIDASE 2A)	up	up	up			H ₂ O ₂
AT3G10300	calcium ion binding	up	up	up	¹ O ₂		
AT3G21080	unknown protein_ ABC transporter-related	up	up	up			H ₂ O ₂
AT4G01010	ATCNGC13; calmodulin binding	up	up	up	¹ O ₂		
AT4G10500	oxidoreductase	up	up	up			H ₂ O ₂
AT4G23150	kinase	up	up	up			H ₂ O ₂
AT4G38550	unknown protein	up	up	up		O ₂ ⁻	
AT5G03350	carbohydrate binding_ legume lectin family protein	up	up	up			H ₂ O ₂
AT5G45380	solute:sodium symporter/ urea transporter	up	up	up			H ₂ O ₂
AT5G56870	beta-galactosidase, putative / lactase, putative	up	up	up			H ₂ O ₂
AT1G20350	ATTIM17-1; protein translocase	up	up		¹ O ₂		
AT1G20510	4-coumarate-CoA ligase	up	up				H ₂ O ₂
AT1G22280	protein phosphatase type 2C	up	up		¹ O ₂		
AT1G23830	unknown protein	up	up				H ₂ O ₂
AT1G28370	ATERF11	up	up				H ₂ O ₂
AT1G52720	unknown protein	up	up		¹ O ₂		

Table 3B. Continued

gene ID	function	mekk1	mpk4	mkk1/2	singlet oxygen	superoxide	H ₂ O ₂
AT2G24160	unknown protein_ pseudogene, leucine rich repeat protein family	up	up				H ₂ O ₂
AT2G25625	unknown protein	up	up		¹ O ₂		
AT2G27830	unknown protein	up	up		¹ O ₂		
AT2G43530	trypsin inhibitor, putative	up	up				H ₂ O ₂
AT2G44380	unknown protein_ DC1 domain-containing protein	up	up		¹ O ₂		
AT2G46620	nucleoside-triphosphatase	up	up		¹ O ₂		
AT2G47130	oxidoreductase	up	up				H ₂ O ₂
AT3G09520	protein binding_ exocyst subunit EXO70 family protein	up	up				H ₂ O ₂
AT3G16720	ATL2 ubiquitin-protein ligase	up	up		¹ O ₂		
AT3G46620	ubiquitin-protein ligase/ zinc ion binding	up	up		¹ O ₂		
AT3G47160	ubiquitin-protein ligase/ zinc ion binding	up	up			O ₂ ⁻	
AT4G03510	RMA1 ubiquitin-protein ligase/ zinc ion binding	up	up			O ₂ ⁻	
AT4G08150	KNAT1 (BREVIPEDICELLUS 1); transcr factor_ homeobox protein	up	up		¹ O ₂		
AT1G33420	DNA binding / protein binding / zinc ion binding	up		up			H ₂ O ₂
AT2G28110	transferase_ exostosin family protein	up		up			H ₂ O ₂
AT2G47190	MYB2	up		up			H ₂ O ₂
AT3G28210	PMZ_ zinc finger (AN1-like) family protein	up		up			
AT3G47340	ASN1 (DARK INDUCIBLE 6)_ asparagine synthetase 1	up		up		O ₂ ⁻	
AT3G54100	unknown protein	up		up	¹ O ₂		
AT4G27940	binding / transporter_ mitochondrial substrate carrier family protein	up		up	¹ O ₂		
AT5G49360	BXL1 (BETA-XYLOSIDASE 1); hydrolase	up		up			H ₂ O ₂
AT1G02660	triacylglycerol lipase	up			¹ O ₂		
AT1G07890	APX1; L-ascorbate peroxidase 1	up					H ₂ O ₂
AT1G19200	unknown protein_ senescence-associated protein-related	up					H ₂ O ₂
AT1G19310	ubiquitin-protein ligase/ zinc ion binding	up			¹ O ₂		
AT1G51790	leucine-rich repeat protein kinase, putative	up			¹ O ₂		
AT1G70140	ATFH8; actin binding (formin homology 2 domain)	up					H ₂ O ₂
AT1G78080	RAP2.4 AP2 domain-containing transcription factor	up			¹ O ₂		
AT2G23450	kinase	up			¹ O ₂		
AT3G21780	UDP-glycosyltransferase	up					H ₂ O ₂
AT3G25600	calcium ion binding_ calmodulin, putative	up			¹ O ₂		
AT3G28600	nucleoside-triphosphatase	up			¹ O ₂		
AT3G29250	oxidoreductase	up			¹ O ₂		
AT3G46280	unknown protein_ protein kinase-related	up				O ₂ ⁻	
AT3G50280	transferase family protein	up					H ₂ O ₂
AT4G08950	phosphate-responsive protein, putative (EXO)	up					H ₂ O ₂
AT4G26400	ubiquitin-protein ligase/ zinc ion binding	up			¹ O ₂		
AT5G54960	PDC2 (PYRUVATE DECARBOXYLASE-2)	up				O ₂ ⁻	
AT5G59780	MYB59	up					H ₂ O ₂
AT1G32450	proton-dependent oligopeptide transport (POT) family protein		down	down	¹ O ₂		
AT3G15950	unknown protein_ DNA topoisomerase-related		down			O ₂ ⁻	
AT3G08770	LTP6lipid transfer protein 6		up	down		O ₂ ⁻	
AT2G15080	kinase_ disease resistance family protein		up	up			H ₂ O ₂
AT5G44070	CAD1 (CADMIUM SENSITIVE 1)_ phytochelatin synthase 1 (PCS1)		up	up	¹ O ₂		
AT1G10850	leucine-rich repeat transmembrane protein kinase, putative		up		¹ O ₂		

Table 3B. Continued

gene ID	function	mekk1	mpk4	mkk1/2	singlet oxygen	superoxide	H ₂ O ₂
AT1G58420	unknown protein		up		¹ O ₂		
AT1G59870	ABC transporter family protein		up		¹ O ₂		
AT3G17110	unknown protein_ pseudogene, glycine-rich protein		up		¹ O ₂		
AT4G22490	lipid transfer protein (LTP) family protein		up			O ₂ ⁻	
AT4G36500	unknown protein		up		¹ O ₂		
AT1G05840	pepsin A_ aspartyl protease family protein			down			H ₂ O ₂
AT1G18250	ATLP-1_ thaumatin, putative			down		O ₂ ⁻	
AT1G28290	pollen Ole e 1 allergen and extensin family protein			down		O ₂ ⁻	
AT1G68875	unknown protein			down	¹ O ₂		
AT1G73330	ATDR4_ protease inhibitor, putative (DR4)			down		O ₂ ⁻	
AT1G75910	EXL4; acyltransferase			down	¹ O ₂		
AT1G75940	ATA27 hydrolase anther-specific protein			down	¹ O ₂		
AT2G21660	ATGRP7; RNA binding_ glycine-rich RNA-binding protein			down		O ₂ ⁻	
AT2G28790	unknown protein_ osmotin-like protein, putative			down		O ₂ ⁻	
AT2G31980	cysteine protease inhibitor			down	¹ O ₂		
AT2G41650	unknown protein			down			H ₂ O ₂
AT3G02640	unknown protein			down		O ₂ ⁻	
AT3G15400	ATA20_ anther development protein, putative			down	¹ O ₂		
AT3G21770	peroxidase 30 (PER30) (P30) (PRXR9)			down		O ₂ ⁻	
AT3G50970	dehydrin xero2 / low-temperature-induced protein LTI30			down	¹ O ₂		
AT3G57010	strictosidine synthase			down			H ₂ O ₂
AT4G15910	ATDI21 drought-induced protein (Di21)			down		O ₂ ⁻	
AT4G27860	unknown protein_ integral membrane family protein			down	¹ O ₂		H ₂ O ₂
AT4G29030	unknown protein_ glycine-rich protein			down		O ₂ ⁻	
AT4G29700	nucleotide pyrophosphatase family protein			down	¹ O ₂		
AT4G37410	CYP81F4			down		O ₂ ⁻	H ₂ O ₂
AT5G07550	GRP19_ glycine-rich protein			down	¹ O ₂		
AT5G07560	GRP20; nutrient reservoir_ glycine-rich protein			down	¹ O ₂		
AT5G17220	ATGSTF12 (GLUTATHIONE S-TRANSFERASE 26)			down		O ₂ ⁻	H ₂ O ₂
AT5G50790	unknown protein_ nodulin MtN3 family protein			down	¹ O ₂		
AT1G28380	unknown protein			up	¹ O ₂		
AT1G74100	sulfotransferase_ sulfotransferase family protein			up	¹ O ₂		
AT1G74450	unknown protein			up	¹ O ₂		
AT2G22300	ethylene-responsive calmodulin-binding protein, putative (SR1)			up	¹ O ₂		
AT3G07040	RPM1 (RESISTANCE TO P. SYRINGAE PV MACULICOLA 1)			up			H ₂ O ₂
AT3G47250	unknown protein			up			H ₂ O ₂
AT4G23800	WRKY53			up		O ₂ ⁻	

Table 3C. Genes Responsive to Multiple or to a Given Type of ROS and their Altered Expression in *mekk1*, *mkk1/2*, and *mpk4* Mutants.

	Response to multiple types of ROS		response to specific ROS type		
	total	transcription factors	singlet oxygen	superoxide	H ₂ O ₂
total number of genes identified by Gadjev et al., (2006)	103	32	314	194	193
misregulated in <i>mekk1</i> , <i>mkk1/2</i> and/or <i>mpk4</i> mutants	46	20	48	31	43
proportion	45%	63%	15%	15%	22%

Total number of responsive genes as identified by Gadjev et al. (2006) compared to their abundance in the differential transcriptomes of *mekk1*, *mkk1/2*, and/or *mpk4* mutants. For details, see Tables 3A and 3B.

Table 4. Altered Expression of ROS Scavenging Enzyme-Encoding Genes in *mekk1*, *mkk1/2*, and/or *mpk4* Mutants.

function	gene ID	localisation	mekk1	mpk4	mkk1/2	reaction
Blue copper binding protein	At5g20230	sec	up	up	up	$\text{Cu} + \text{P} \rightarrow \text{P-Cu}$
NADPH oxidase (RbohL)	At4g11230	mem	up	up	up	$\text{NADPH} + \text{e}^- + \text{O}_2 \rightarrow \text{NADP}^+ + \text{O}_2^- + \text{H}^+$
Glutaredoxin family	At1g06830	sec/mit	up	up	up	$\text{DHA} + 2 \text{GSH} \rightarrow \text{Asc} + \text{GSSG}$
Glutaredoxin family	At1g28480	cyt/mit	up	up	up	$\text{DHA} + 2 \text{GSH} \rightarrow \text{Asc} + \text{GSSG}$
Cu/ZnSOD (CSD1)	At1g08830	cyt	up	up		$\text{O}_2^- + \text{O}_2^- + 2\text{H}^+ \rightarrow \text{H}_2\text{O}_2 + \text{O}_2$
Thioredoxin H-type 5 (TRX-H-5)	At1g45145	cyt	up	up		$\text{P-S-S-P} + 2\text{H}^+ \rightarrow 2\text{P-SH}$
Glutaredoxin putative	At1g77370	sec	up	up		$\text{DHA} + 2 \text{GSH} \rightarrow \text{Asc} + \text{GSSG}$
Glutaredoxin putative	At5g63030	cyt	up	up		$\text{DHA} + 2 \text{GSH} \rightarrow \text{Asc} + \text{GSSG}$
Cat1	At1g20630	per	up		up	$2\text{H}_2\text{O}_2 \rightarrow 2\text{H}_2\text{O} + \text{O}_2$
Cat3	At1g20620	per	up		up	$2\text{H}_2\text{O}_2 \rightarrow 2\text{H}_2\text{O} + \text{O}_2$
APX1	At1g07890	cyt	up			$2 \text{Asc} + \text{H}_2\text{O}_2 \rightarrow 2 \text{MDA} + 2\text{H}_2\text{O}$
GPX5	At3g63080	er	up			$\text{H}_2\text{O}_2 + 2 \text{GSH} \rightarrow 2\text{H}_2\text{O} + \text{GSSG}$
thylakoid-APX	At1g77490	chl	down	down	down	$2 \text{Asc} + \text{H}_2\text{O}_2 \rightarrow 2 \text{MDA} + 2\text{H}_2\text{O}$
Ferric-chelate reductase	At1g01580		down	down	down	$\text{NADPH} + \text{e}^- + \text{O}_2 \rightarrow \text{NADP}^+ + \text{O}_2^- + \text{H}^+ (?)$
Cat2	At4g35090	per	down	down		$2\text{H}_2\text{O}_2 \rightarrow 2\text{H}_2\text{O} + \text{O}_2$
Ferredoxin-thioredoxin reductase	At2g04700	chl	down	down		$\text{P-S-S-P} + 2\text{H}^+ \rightarrow 2\text{P-SH}$
Blue copper protein, putative	At1g72230	sec	down		down	$\text{Cu} + \text{P} \rightarrow \text{P-Cu}$
Blue copper-binding protein, 15K (lamin)	At4g12880	sec	down		down	$\text{Cu} + \text{P} \rightarrow \text{P-Cu}$
APX4	At4g09010	chl	down			$2 \text{Asc} + \text{H}_2\text{O}_2 \rightarrow 2 \text{MDA} + 2\text{H}_2\text{O}$
GR2	At3g54660	chl/mit	down			$\text{GSSG} + \text{NAD(P)H} \rightarrow 2 \text{GSH} + \text{NAD(P)}^-$
2-cys PrxR A	At3g11630	chl	down			$\text{GSSG} + \text{NAD(P)H} \rightarrow 2 \text{GSH} + \text{NAD(P)}^-$
Thioredoxin H-type 4 (TRX-H-4)	At1g19730	cyt		up		$\text{P-S-S-P} + 2\text{H}^+ \rightarrow 2\text{P-SH}$
Glutaredoxin family	At2g47880	sec/mit			down	$\text{DHA} + 2 \text{GSH} \rightarrow \text{Asc} + \text{GSSG}$

gene	differential in mekk1, mkk1/2 and/or mpk4	genes in family	reaction
Superoxide Dismutase (SOD)	1	8	$\text{O}_2^- + \text{O}_2^- + 2\text{H}^+ \rightarrow \text{H}_2\text{O}_2 + \text{O}_2$
Ascorbate Peroxidase (APX)	3	9	$2 \text{Asc} + \text{H}_2\text{O}_2 \rightarrow 2 \text{MDA} + 2\text{H}_2\text{O}$
Monodehydroascorbate Reductase (MDAR)	0	5	$\text{MDA} + \text{NAD(P)H} + \text{H}^+ \rightarrow \text{Asc} + \text{NAD(P)}^-$
Dehydroascorbate Reductase (DHAR)	0	5	$\text{DHA} + 2 \text{GSH} \rightarrow \text{Asc} + \text{GSSG}$
Glutathione Reductase (GR)	0	2	$\text{GSSG} + \text{NAD(P)H} \rightarrow 2 \text{GSH} + \text{NAD(P)}^-$
Catalase (Cat)	1	3	$2\text{H}_2\text{O}_2 \rightarrow 2\text{H}_2\text{O} + \text{O}_2$
Glutathione Peroxidase (GPX)	0	8	$\text{H}_2\text{O}_2 + 2 \text{GSH} \rightarrow 2\text{H}_2\text{O} + \text{GSSG}$
Ferritin	0	4	$\text{Fe} + \text{P} \rightarrow \text{P-Fe}$
Blue copper protein	3	9	$\text{Cu} + \text{P} \rightarrow \text{P-Cu}$
NADPH oxidase	1	10	$\text{NADPH} + \text{e}^- + \text{O}_2 \rightarrow \text{NADP}^- + \text{O}_2^- + \text{H}^+$
NADPH oxidase-like	1	9	$\text{NADPH} + \text{e}^- + \text{O}_2 \rightarrow \text{NADP}^- + \text{O}_2^- + \text{H}^+ (?)$
Alternative Oxidase (AOX)	0	6	$2\text{e}^- + 2\text{H}^+ + \text{O}_2 \rightarrow \text{H}_2\text{O}$
Peroxiredoxin (PrxR)	1	11	$2\text{P-SH} + \text{H}_2\text{O}_2 \rightarrow \text{P-S-S-P} + 2\text{H}_2\text{O}$
Thioredoxins (Trx)	3	32	$\text{P-S-S-P} + 2\text{H}^+ \rightarrow 2\text{P-SH}$
Glutaredoxin (GLR)	5	27	$\text{DHA} + \text{GSH} \rightarrow \text{Asc} + \text{GSSG}$
total	23	148	

H_2O_2 in *mekk1* and *mpk4* plants as has been observed by staining of mutants with the H_2O_2 -specific dye DAB (Nakagami et al., 2006). Surprisingly, the steady-state transcript levels of *CAT1* and *CAT3* are elevated in *mekk1*, and also in *mkk1/2*, but not in *mpk4*. The simultaneous repression of *CAT2* and induction of *CAT1* and *CAT3* in *mekk1* might point to a feedback regulatory mechanism—an ‘attempt’ of these

mutants to compensate for insufficient *CAT2* levels by enhancing *CAT1* and *CAT3* synthesis. That such feedback mechanisms might exist is supported by a study investigating the kinetics and localization of catalase gene expression in *Arabidopsis* during plant development (Zimmermann et al., 2006). The expression and activity of *CAT2*, which are specific for photosynthetically active tissue, decrease during senescence before

a detectable loss of chlorophyll. *CAT2* down-regulation is assumed to be the initial step in the H_2O_2 peak during bolting time, whereas the decrease in APX1 (ascorbate peroxidase) activity presumably is only a secondary and amplifying effect (Zimmermann et al., 2006). *CAT3* displays vasculature-specific expression, is induced with age and corresponds to an accumulation of H_2O_2 in the vascular bundles. The H_2O_2 peak resulting from senescence-induced *CAT2* down-regulation results in *CAT3* enzyme activation, followed by a recovery of APX1 activity and subsequent decline of H_2O_2 (Zimmermann et al., 2006). The lack of *CAT3* induction specifically in *mpk4*, but not in *mekk1* and *mkk1/2* mutants despite high H_2O_2 levels, suggests a role of MPK4 in the sensing of vascular H_2O_2 independently of MEKK1 and MKK1/MKK2. Likewise, the down-regulation of *CAT2* in *mekk1* and *mpk4*, but not *mkk1/2*, mutants indicates (1) that disturbances of additional redoxregulatory mechanisms other than repression of *CAT2* account for the H_2O_2 accumulation in *mkk1/2* mutant plants and (2) that *CAT2* expression is controlled by a MEKK1–MPK4 pathway bypassing MKK1/MKK2.

Regulation of Photosynthesis by MEKK1–MPK4

A regulated appropriate balance of photosynthetic input of H_2O and CO_2 and removal of O_2 is necessary for optimal photosynthesis. Under various suboptimal conditions, excess excitation energy initiates dangerous ROS production. A connection of MAPK-regulated ROS control of photosynthesis is provided through a recent report showing that activation of three tobacco MAPKs, SIPK/Ntf4/WIPK by DEX-induced expression of a constitutively active MAPKK (MEK2DD) rapidly inhibits photosynthesis concurrently with strong accumulation of H_2O_2 (Liu et al., 2007). More detailed studies revealed that MEK2DD-induced shutdown of carbon fixation triggers chloroplastic superoxide production, which is rapidly converted

to H_2O_2 by superoxide dismutase. Prolonged activation of the MAPK cascade (10 h) leads to chloroplast damage in a light-dependent manner, resulting in an HR-like cell death. Also ROS generation upon challenge of plants with tobacco mosaic virus (TMV), which activates SIPK and WIPK (Zhang and Klessig, 1998), is greatly enhanced upon light. This observation is similar to the finding that incompatible pathogen-induced HR cell death is light-dependent (Bechtold et al., 2005). Examination of the kinetics of carbon fixation inhibition and onset of cell death upon *MEK2DD* expression indicates that the MAPK cascade actively blocks carbon fixation, leading to an excess of excitation energy in illuminated plants and—consequently—accumulation of ROS. The sustained generation of ROS cannot be compensated for by the action of antioxidant enzymes, and, after depletion of the antioxidant pool, leads to cell death (Liu et al., 2007).

Interestingly, among the genes down-regulated specifically in *mekk1* and *mpk4*, but not in *mkk1/2*, mutants, those encoding plastidic or chloroplastic proteins are significantly overrepresented (Table 5), suggesting that photosynthetic suppression of genes through MEKK1 and MPK4 bypasses MKK1/MKK2. These results would suggest that MEKK1-dependent activation of MPK4 might also occur independently or through other MAPKKs. In this context, it should be noted that in yeast two-hybrid assays, MEKK1 was found to directly interact with MPK4 (Ichimura et al., 1998). Biochemical analysis later confirmed the specificity of the MEKK1–MPK4 interaction (Nakagami et al., 2006). It is presently unclear whether MEKK1 can activate MPK4 directly or whether MAPKKs other than MKK1 and MKK2 can function in MPK4 activation.

MEKK1-Specific Genes

Although a considerable number of differentially expressed genes is shared between *mekk1*, *mkk1/2*, and *mpk4* mutants

Table 5. Abundance of Selected Annotations of Genes Differentially Regulated in *mekk1*, *mkk1/2*, and/or *mpk4* Mutants Compared to the Overall Abundance in the *Arabidopsis* Genome.

	up				down				
	mekk1	mekk1 mkk1/2	mekk1 mpk4	mekk1 mkk1/2 mpk4	mekk1	mekk1 mkk1/2	mekk1 mpk4	mekk1 mkk1/2 mpk4	
% genome									
Total number of differentially expressed genes	306	184	186	235	388	127	151	49	
chloroplast	23	14	11	17	100	18	49	4	
%	9.83	7.51	7.61	5.91	7.23	25.77	14.17	32.45	8.16
p-value	0.1688	0.3220	0.0760	0.1753	<0.0001	0.1002	<0.0001	0.6951	
plastid	7	3	2	2	43	2	24	3	
%	2.92	2.29	1.63	1.08	0.85	11.08	1.57	15.89	6.12
p-value	0.5055	0.3035	0.1374	0.0586	<0.0001	0.3679	<0.0001	0.1830	
response to stress	37	30	22	49	37	14	20	5	
%	6.6	12.05	16.39	11.82	20.85	9.54	11.02	13.24	10.2
p-value	<0.0001	<0.0001	0.0037	<0.0001	0.0198	0.0447	0.0010	0.3096	

P-values of Chi-square test are shown. Statistically significant values are shaded. Gene annotations were retrieved from the TAIR webtool.

(284 of a total of 1635, 1142, and 776, respectively), the transcriptome analysis of these mutants also revealed that expression of a large set of genes (694 of 1635) is only affected in *mekk1* mutants (Figure 1). Within this pool of *mekk1*-specific genes, several SA- and ROS-responsive genes are found. Their proportion is not significantly different from that in the datasets of genes showing altered transcript levels also in *mkk1/2* and/or *mpk4*.

These data suggest that:

- (1) MEKK1 is the upstream activator of MKK1/MKK2 and MPK4;
- (2) MEKK1 activates an independent pathway, not involving MKK1/MKK2 or MPK4;
- (3) MKK1/2 is not only regulating MPK4, but most likely also other MAPKs (MPK6?);
- (4) MEKK1 activates MPK4 to some extent in an MKK1/MKK2-independent manner (Figure 2);
- (5) some SA signalling might be regulated by MEKK1 via a MKK1/MKK2- and MPK4-independent mechanism.

We cannot exclude that some of the alterations in ROS gene expression are due to defective developmental programs in the mutants. Also, other non-overlapping roles of MEKK1, MKK1/2, and MPK4 might exist that would explain why some ROS-responsive genes are unaffected in one mutant line but differentially expressed in the other mutant lines.

The spontaneous HR-like cell death in the mutants can be the trigger of differential gene expression of some ROS genes, while it might be the consequence of differential gene expression of other ROS genes. It has been reported that *mekk1* and *mpk4* mutants, when grown at higher temperatures, develop normally; and this coincides with a decrease of the SA accumulation in these mutants.

A comparison of the transcriptome of the mutants grown at elevated temperature, and a subsequent shift to normal (i.e. HR-supporting) temperature would help to distinguish between direct and secondary effects of the MAPK components on ROS gene expression.

Feed-Forward and Feed-Back Loops in MAPK Signalling

With an increasing number of microarray data becoming publicly available, a more and more complex pattern of MAPK regulation arises. Not only are MAPK cascade components post-translationally activated in the process of signal transduction from receptor to MAPK-targeted effector, but genes encoding several MAPK pathway components are subject to transcriptional regulation themselves. For instance, in a search for genes whose expression is rapidly induced upon wounding (5 min), MPKK9 and MPK3 as well as *AP2C1*, a PP2C-type phosphatase with a MAPK interaction motif, have been identified (Walley et al., 2007), indicating that transcriptional control of both phosphorylation and dephosphorylation of MAPK signalling components is involved in transduction of initial stress signalling events.

These data suggest that our 'classical' signalling concepts are oversimplified and multiple feed-forward and feed-back

mechanisms are involved so that gene expression might be part of the signalling process itself. A good example might be provided by MPK3. The fast kinetics of MPK3 activation upon a series of challenging conditions (Walley et al., 2007; Pitzschke and Hirt, 2006; Nakagami et al., 2005) suggests that, initially, already existing MPK3 protein is being used for signal transduction. The stress-induced accumulation of *MPK3* transcripts over an extended period, as has been observed in several gene expression studies (Mizoguchi et al., 1996; Wan et al., 2004; Walley et al., 2007), might be indicative of the need for a continuous supply of MPK3 enzyme, which would feed into the cascade and thereby contribute to amplifying the stress signal. Interestingly, in contrast to MPK3, its closest homolog, MPK6, underlies neither transcriptional nor translational control (Ulm et al., 2002). Why enhanced levels of some MAPK components persist long after MAPK activation has declined is a puzzling question.

An even more complex scenario of MAPK-regulated gene expression became apparent from the isolation of the *Arabidopsis* MAPKKK MEKK1 from a screen for proteins binding to the promoter of the *WRKY53* gene. *WRKY53* is a member of the plant-specific transcription factor family of WRKYs, which, in *A. thaliana*, comprises 74 members, many of which are transcriptionally inducible upon pathogen infection and other defence-related stimuli (Dong et al., 2003; Kalde et al., 2003). MEKK1 does not only interact with the *WRKY53* promoter, but also binds to and phosphorylates the *WRKY53* gene product (Miao et al., 2007). Previous studies had shown *WRKY53* to induce the expression of stress and defence-related as well as senescence-associated genes (Miao et al., 2004). Characterization of the *WRKY53* promoter region bound by MEKK1 revealed that it is important for the switch of *WRKY53* expression from a leaf age-dependent to a systemic plant age-dependent expression during bolting. As *in-vitro* analysis and studies with a *WRKY53* promoter-driven reporter gene have shown, MEKK1 binding to and phosphorylation of *WRKY53* enhances the DNA-binding capacity of *WRKY53* (Miao et al., 2007). *WRKY53* expression is induced by H₂O₂ (Miao et al., 2004), and results from transient expression studies with *WRKY53* promoter deletion constructs suggest MEKK1 to be involved in the hydrogen peroxide response of *WRKY53* (Miao et al., 2007).

The apparent short-cut in mitogen-activated protein kinase (MAPK) signalling by direct phosphorylation of a transcription factor through a MAPKKK is so far unique to MEKK1. *WRKY53* expression is also induced upon treatment with the fungal cell wall-derived elicitor chitin (review Montesano et al., 2003) known to activate MPK3 and MPK6 (Nühse et al., 2000; Gust et al., 2007), as well as by ectopic expression of the tobacco MAPKK NtMEK2 active mutant *NtMEK2^{DD}* (Wan et al., 2004). We also found *WRKY53* within the dataset of genes commonly up-regulated in *mekk1*, *mkk1/2*, and *mpk4*. Together, these data suggest that *WRKY53* expression is not only controlled by the MEKK1 short-cut but most likely also through classical MAPK signalling pathways.

DISCUSSION

Over the last couple of years, considerable efforts have been undertaken to disentangle the networks regulating basal ROS levels and targeted ROS synthesis in plants. Several MAPK cascade components were found to be involved, often in more than one aspect/process of ROS signalling. Clearly, MAPK pathways play a key role in controlling normal development and dynamic processes, such as flower development, stomatal patterning, and stomatal aperture, and are thus indispensable for ROS homeostasis. MAPK(KK) activities are regulated by a sophisticated network involving transcriptional, translational and posttranslational control as well as a diverse pattern of feedback loops. In a number of very recent studies, additional fascinating features of stress-response or developmental regulation of and through MAPK(KK)s have been identified. Our present analysis contributes to the understanding of redox-regulation through MAPKs. The strong overlap of the transcriptomes of *mekk1*, *mkk1/2*, and *mpk4* mutants and the reported stress-responsiveness of the corresponding genes plausibly shows the existence of a full MAPK cascade (MEKK1–MKK1/MKK2–MPK4) as a key regulator of ROS- and SA-initiated stress signalling. Surely, many more astonishing scenarios of MAPK-controlled ROS production and ROS-controlled MAPK activity will be disclosed in the future.

METHODS

Plant Material

Surface-sterilized seeds were sown on agar plates containing half-strength MS medium, incubated at 4°C for 2 d and subsequently transferred to a controlled-environment room (22°C, 8 h photoperiod).

Ecotype Columbia *mekk1*, *mpk4* (Nakagami et al., 2006), *mkk1* (Mészáros et al., 2006) and *mkk2* mutant lines (Teige et al., 2004) have been described previously.

Homozygous *mkk1* and *mkk2* mutant plants were crossed. Two plants carrying both T-DNA insertions were identified by genotyping. Genotyping of the progeny (30 individuals) of these plants showed that dwarfed individuals were homozygous for both insertions.

Fourteen-day-old seedlings for microarray analysis were selected based on their dwarfed phenotype. An aliquot of the RNA extracted for microarray analysis was used to confirm via RT-PCR that the harvested material was indeed homozygous for the respective insertions.

Transcriptome Studies

The microarray analysis was performed using the CATMA array containing 24 576 gene-specific tags (GSTs) corresponding to 22 089 genes, including 21 612 AGI-predicted and 477 Eugene-predicted genes (Crowe et al., 2003; Hilson et al., 2004). For each comparison (mutant line versus Col-O wild-type), we performed a biological repeat using an independent second set of samples. In addition, to avoid dye bias and gene-

specific dye bias, a dye-swap experiment was carried out. Therefore, four arrays were used for each comparison assay. For each sample, total RNA was extracted from pools of 50 plantlets using the RNeasy Plant Mini Kit (Qiagen) following the manufacturer's protocol. RNA integrity, cDNA synthesis, hybridization, and array scanning were performed as described by Lurin et al. (2004).

Statistical Treatment of Microarray Data

Normalization and statistical analysis were based on two dye swaps, i.e. four arrays, as described in Gagnot et al. (2008). To determine differentially expressed genes, we performed a paired *t*-test on the log ratios, assuming that the variance of the log ratios was the same for all genes. Spots displaying extreme variance (too small or too large) were excluded. The raw *P*-values were adjusted by the Bonferroni method, which controls the Family Wise Error Rate. We use the Bonferroni method (with a type I error equal to 5%) in order to keep a strong control of the false positives in a multiple-comparison context (Ge et al., 2003). We considered as being differentially expressed the genes with a Bonferroni *P*-value ≤ 0.05 , as described in Gagnot et al. (2008).

Microarray data from this article were deposited at Gene Expression Omnibus (www.ncbi.nlm.nih.gov/geo/; accession no. GSE10763/GSE10764) and at CATdb (<http://urgv.evry.inra.fr/CATdb/>; Project: ADT06-03_MKK2-cold) according to the Minimum Information About a Microarray Experiment standards.

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