Functional Analysis of a Novel MAP Kinase Cascade in Jasmonate Signaling in *Arabidopsis thaliana*

January 2007

Fuminori TAKAHASHI

Functional Analysis of a Novel MAP Kinase Cascade in Jasmonate Signaling in *Arabidopsis thaliana*

A Dissertation Submitted to

the Graduate School of Life and Environmental Sciences,

the University of Tsukuba

in Partial Fulfillment of the Requirements

for the Degree of Doctor of Philosophy in Science

(Doctoral Program in Functional Biosciences)

Fuminori TAKAHASHI

Table of Contents

Results

Abbreviations

ACC : 1-aminocyclopropane-1-carboxylic acid

ACO : ACC oxidase

ACS : 1-aminocyclopropane-1-carboxylic acid synthase

At : *Arabidopsis thaliana*

AUX : auxin

CBB : Coomassie brilliant blue

Da : dalton

β-CHI : *β-chitinase*

COI1 : *coronatine insensitive mutant 1*

CTR1 : *constitutive triple response 1*

DAB : diaminobenzidine

DEX : dexamethazone

DTT : dithiothreitol

ECL : enhanced chemiluminescence

EDTA : ethylenediaminetetraacetic acid

EGTA : ethylene glycol bis(2-aminoethyl-ether) tetraacetic acid

EDS1 : *enhanced disease susceptibility 1*

ein : *ethylene insensitive mutant*

ERF1 : *ethylene response factor 1*

ET : ethylene

FRK1 : *flg22-induced receptor-like kinase 1*

F.W. : fresh weight

g : centrifugal force (x unit gravitational field)

GST : glutathion S-transferase

HR-like : hypersensitive response-like

IP-kinase : immunocomplex kinase

IPTG : isopropyl-β-D-thiogalactopyranoside

JA : jasmonic acid

JR : *JA-responsive*

kbp : kilo base pairs

Le : *Lycopersicon esculentum*

LRR : leucine rich repeat

MAPK : mitogen-activated protein kinase

MAPKK : MAPK kinase

MAPKKK : MAPKK kinase

MBP : myelin basic protein

MeJA : methyl jasmonate

MEK : MAPK/Erk kinase

MEKK : MEK kinase

MKS1 : MAPK substrate 1

Ms : *Medicago sativa*

NPK : *Nicotiana* protein kinase

Nt : *Nicotiana tobacum*

NTF2 : nuclear transport factor 2

Os : *Oryza sativa*

PAD : phytoalexin-deficient

PAGE : polyacrylamid gel electrophoresis

PAMP : pathogen-associated molecular pattern

PBS : phosphate-buffered saline

PCR : polymerase chain reaction

PDF1.2 : *plant defensin 1.2*

PI : proteinase inhibitors

PMSF : phenyl methyl sulfonyl fluolide

PR protein : pathogenesis-related protein

QRT-PCR : quantitative RT-PCR

ROS : reactive oxygen species

RT-PCR : reverse transcriptase-PCR

SAR : systemic acquired resistance

SDS : sodium dodecyl sulfate

SIPK : salicylic acid-induced protein kinase

Ss : *Suaeda salsa*

St : *Solanum tuberosum*

T-DNA : transferred DNA

Thi2.1 : *thionin gene 2.1*

Va : *Vitis aestivalis*

VSP : *vegetative storage protein*

WIPK : wound-induced protein kinase

Zm : *Zea mays*

Abstract

Plant responses to various types of biotic/abiotic stresses are regulated locally and systemically by signaling molecules such as jasmonic acid (JA). JA regulates such diverse processes as wound responses and pollen maturation. Protein phosphorylation/dephosphorylation has a key role in the signal transduction pathways under various stress conditions. Based on the studies using protein kinase and phosphatase inhibitors, protein phosphorylation and dephosphorylation are implicated in the regulation of JA-inducible gene expression; however, actual components of JA signaling remain largely unknown. Mitogen-activated protein kinase (MAPK) cascade is one of the major signal transduction pathways in plants as well as other eukaryotes. In *Arabidopsis thaliana*, a large number of MAPK families exist in its genome. However, it has been still unknown how these gene products compose the MAPK cascades and function as biological signal mediators. Therefore, identification and functional analysis of novel MAPK cascade are important to understand the signal transduction mechanism in *Arabidopsis*. In general, there is the substrate specificity between MAPK kinase (MAPKK) and MAPK. MAPKK family has 10 members and is less diverse than MAPK and MAPKKK. Therefore, I focused to analyze MAPKK families to identify novel MAPK cascade.

The *Arabidopsis* MAP kinase kinase 3 (MKK3) is an orphan MAPKK because of its specific structure in all eukaryotes and unidentified function. IN the present study, I demonstrate a novel *Arabidopsis* MAPK cascade, MKK3-MPK6, functioning in JA signaling. First, I established *in vitro* activation assay system using by GST-fusion proteins expressed in *Escherichia coli*, and found that MKK3 specifically activates MPK6. I also demonstrated that MKK3-MPK6 cascade exists *in planta* by using dexamethazone (DEX)-mediated conditional expression transgenic

system. The *MKK3* and *MPK6* were expressed in various tissues in *Arabidopsis*. These results suggest that a novel MKK3-MPK6 cascade functions as well as reported MKK4-MPK6 cascade in *Arabidopsis*.

Substitution of serine (S) and/or threonine (T) in the activation loop of MAPKK for aspirate (D) is known to constitutively activate MAPKKs. DEX-treated *MKK4DD* plants produced ethylene (ET), and showed the ET-mediated morphological phenotype. On the other hand, DEX-treated *MKK3DD* plants showed neither the ET accumulation nor triple response phenotype. *MKK3DD* plants did not show HR-like cell death phenotype as those found in *MKK4DD* plants. In addition, the occasion of hypersensitive response-like (HR-like) cell death phenotype in *MKK4DD* plants was due to the generation of reactive oxygen species (ROS) based on the analysis by diaminobenzidine (DAB) stain system. However, *MKK3DD* plants did not generate ROS production. These results suggest that the MKK3-MPK6 cascade is involved in different biological process other than that of the MKK4-MPK6, although both MKK3 and MKK4 activate MPK6.

For exploring the biological function of the MKK3-MPK6 cascade, I analyzed the downstream gene expression by using microarray analysis, and found that the MKK3-MPK6 cascade is involved JA signaling. Exogenous application of JA activated MPK6, and this activation was severely impaired in the *MKK3*-knockout mutant *mkk3*, and JA-insensitive mutant *coi1*. Plants overexpressing either *MKK3* or *MPK6* were insensitive to JA in root growth. In contrast, both *mkk3* and *mpk6* plants showed increased sensitivity to JA in root growth. These results indicate that the MKK3-MPK6 cascade mediates JA signaling.

Previous studies provide evidence for positive interactions between the JA

and ET signaling pathways. Both JA and ET signaling are required for the expression of the defense-related gene *PDF1.2* in response to infection by *Alternaria brassicicola*. In transgenic plants overexpressing either *MKK3* or *MPK6*, the expression of *PDF1.2* by JA treatment was upregulated, whereas, it was downregulated in either *mkk3* or *mpk6* mutant. Therefore, it was assumed that the MKK3-MPK6 cascade may be involved in both JA and ET signaling pathways. However, this regulation was not due to the ET accumulation and the *ERF1* expression in the MKK3-MPK6 cascade. On the contrary, JA-inducible expression of *AtMYC2/JIN1* and *VSP2* genes increased in either *mkk3* or *mpk6* mutants, but reduced in either *MKK3* or *MPK6* overexpressors. Based on the analysis of the JA-mediated root growth inhibition and gene expression in the *mkk3-1*/*atmyc2-3* double mutant, the MKK3-MPK6 cascade was shown to negatively regulate JA-inducible expression of *AtMYC2*. These results indicate that the MKK3-MPK6 cascade plays a crucial role in JA signaling and that its positive and negative regulations by JA may be involved in the *AtMYC2/JIN1* expression in *Arabidopsis*. I propose a model explaining how a MAPK, MPK6, can convert three distinct signals, JA, pathogen, and cold/salt stress into three different sets of responses in *Arabidopsis*, and the roles of upstream MAPKKs to activate MPK6 in these different signaling pathways.

Introduction

Plants have an ability to cope with an unfavorable external environment, for instance biotic and abiotic stresses like any other living organism. Plants perceive those stresses and immediately activate signaling machinery including gene expression to protect them by changing their physiological status including gene expression. One of the universal signaling modules functioning in response to such external stimuli is the MAPK cascade. The MAPK cascade is evolutionary conserved among the eukaryotes and typically consists of three kinds of protein kinases, MAPK, MAPKK, and MAPKK kinase (MAPKKK) (Chen et al., 2001). In this phosphorylation cascade, the MAPKKK, a serine/threonine kinase, is phosphorylated by MAPKKKs, which, in turn, perform threonine and tyrosine dual phosphorylation of MAPKs. This activation occurs within one to several minutes, representing one of the earliest responses against various stimuli (Madhani and Fink, 1998). By responding to external stimuli, the MAPK cascade is activated to play a crucial role in gene expression, metabolism, cell death, proliferation and differentiation in animals and yeasts (Widmann et al., 1999; Chen et al., 2001). In plants, the MAPK cascade is also involved in various biotic and abiotic stress responses, hormone responses, cell division, and developmental processes (Nakagami et al., 2005). Complete sequencing of the *Arabidopsis* genome has revealed the outline of MAPK family that consists of more than 60 MAPKKKs, 10 MAPKKs and 20 MAPKs genes (Ichimura et al., 2002). Despite the large number of MAPKs, only three MAPKs, MPK3, MPK4, and MPK6, have mainly been studied so far. Biochemical analysis revealed that biotic and abiotic stresses such as wounding, touch, cold, salt, osmotic shock, elicitors, and active oxygen spices activated those MAPKs (Nakagami et al., 2005). Recently, reverse genetic approaches have been employed to elucidate the *in planta* function of those MAPKs. MPK4 is a negative

regulator of systemic acquired resistance (SAR) and a positive regulator of *plant defensin 1.2* (*PDF1.2*) expression through *enhanced disease susceptibility 1* (*EDS1*) and *phytoalexin-deficient mutant 4* (*PAD4*), essential components of salicylic acid (SA)-mediated defense responses (Petersen et al., 2000; Brodersen et al., 2006). The substrate protein of MPK4 is MAPK substrate 1 (MKS1; Andreasson et al., 2005). A role of MPK3 in the abscisic acid (ABA)-induced postgermination growth arrest was proposed (Lu et al., 2002). Silencing of MPK6 compromised both gene-for-gene and basal resistance in *Arabidopsis* (Menke et al., 2004). MPK6 and MPK3 appear to be required for ozone tolerance and reciprocally inhibit activity and duration (Miles et al., 2005).

Prevous studies using the constitutive active form of *Arabidopsis* MAPKK have identified downstream MAPKs. For example, MKK4 and MKK5 can activate both MPK3 and MPK6 (Asai et al., 2002; Ren et al., 2002). The 1-aminocyclopropane-1-carboxylic acid synthases ACS2 and ACS6 are substrates for MPK6, and regulated by MPK6 phosphorylation toward ethylene (ET) induction (Liu and Zhang, 2004). This MKK4/MKK5–MPK3/MPK6 cascade also participates in the proposed microbe-associated molecular pattern (MAMP) signaling pathway containing MEKK1 as an MAPKKK (Asai et al., 2002; He et al., 2006). Other approaches such as yeast two-hybrid (Y2H) analysis and yeast complementation suggest that MEKK1 functions upstream of the MKK1/MKK2–MPK4 cascade (Ichimura et al., 1998; Mizoguchi et al., 1998). This cascade plays a role in the MEKK1–MKK2–MPK4/MPK6 cascade during cold and salt stress in *Arabidopsis* (Teige et al., 2004). Several studies strongly suggest that MKK1 is also the upstream factor of MPK4 and mediates bacterial elicitor, wounding, and H_2O_2 signals (Huang et

al., 2000; Matsuoka et al., 2002; Teige et al., 2004). Although these MAPKs are simultaneously activated by many types of stress, MAPK cascades in plants are regulated by complicated upstream factors.

JA is a signaling molecule that plays a key role in the regulation of metabolic processes, reproduction, and environmental stresses responses (Berger, 2002; Devoto and Turner, 2003). JA is rapidly accumulated by wounding, insects attack, and necrotrophic pathogen infection (Turner et al., 2002). In *Arabidopsis*, JA inhibits root elongation, and is required for pollen development, anther dehiscence, and defense against insects and necrotrophic pathogens (Staswick et al., 1992; Feys et al., 1994; McConn et al., 1997; Thomma et al., 1999). JA regulates responses that are by both local and systemic, and which are affected by outputs from signaling pathways regulated by ET, SA, and auxin (Aux; Devoto and Turner, 2003; Farmer et al., 2003). Previous reports revealed that one of the complex cross talk between JA and ET have been shown to either cooperate or antagonize in the regulation of different stress responses, including pathogen attack, wounding, ozone exposure, or exaggerated apical hook development (Devoto and Turner, 2003; Farmer et al., 2003). In *Arabidopsis*, mechanical and biotic wounding activates independent signaling pathways regulating different sets of target genes either at the injury or the systemic site (Leon et al., 2001). JA induces systemic gene expression of vegetative storage protein (*VSP*), JA-responsive gene (*JR1*), and thionin (*Thi2.1*) in response to wounding, but it is negatively regulated by the local synthesis of ET (Rojo et al., 1999). However, JA and ET cooperate synergistically to activate basic pathogenesis-related (PR) proteins such as β-chitinase (*β-CHI*), pathogenesis-related gene (*PR3*), and *PDF1.2* (Xu et al., 1994; Penninckx et al., 1998). Recently, it is suggested that both *ethylene*

response factor 1 (*ERF1*) and *AtMYC2* regulate these cooperation or antagonism between JA and ET. The *ERF1* transcription factor regulates the expression of pathogen-responsive genes dependent on JA/ET pathway (Lorenzo et al., 2003). In *coi1* and *ein2* mutant background, *35S::ERF1* exhibited the reviviscence of defense response and the expression of constitutive activation of JA/ET-regulated genes (Berrocal-Lobo et al., 2002; Lorenzo et al., 2003). Moreover, *ERF1* regulate negatively the induction of wound-responsive genes. On the other hand, analyzing *AtMYC2* knockout mutant, *AtMYC2* regulates positively JA pathway (Boter et al., 2004; Lorenzo et al., 2004). In addition, the expression of *PDF1.2* and *β-CHI* exhibited higher levels in its mutant. These results indicate that there is existence of two branches in the JA signaling (JA/ET pathway and JA pathway) and *ERF1* and *AtMYC2* regulate these pathways antagonistically.

Studies using protein kinase/phosphatase inhibitors have indicated that protein kinase activity may negatively regulate JA-induced systemic gene expression and positively regulate JA/ET-induced local gene expression (Rojo et al., 1998). Transgenic tobacco that overexpresses wound-induced protein kinase (WIPK), a tobacco MPK3 ortholog, shows an increased JA level and constitutive expression of JA-inducible Proteinase inhibitor II (*PI II*; Seo et al., 1999). In tobacco, however, JA activated neither salicylic acid-induced protein kinase (SIPK), a tobacco MPK6 ortholog, nor WIPK (Kumar and Klessig, 2000). Thus, whether JA directly activates MAPK, leading to the induction of local and/or systemic response genes, remains unknown.

Thus far, six MAPKKs (MKK1, 2, 4, 5, 6, and 7) have been identified and analyzed in *Arabidopsis* (Nakagami et al., 2005; Dai et al., 2006). However, MKK3

has not been analyzed phenotypically or biochemically, yet. Here, I describe the functional characterization of *Arabidopsis* MKK3, a group B MAPKK (Ichimura et al., 2002) and an ortholog of tobacco NPK2 (Shibata et al., 1995), which was the first MAPKK isolated from plants. MKK3 has a unique domain composition with a carboxy-terminal domain similar to nuclear transport factor 2 (NTF2; Ichimura et al., 2002). Biochemical analyses in *in planta* and *in vitro* showed that MPK6 is a downstream target of MKK3. Microarray analysis revealed that MKK3 plays a role in JA signaling. Indeed, MPK6 was activated by JA, and this activation depended largely on the MKK3 manner. Loss-of-function and gain-of-function analyses *in planta* revealed that the MKK3–MPK6 cascade negatively regulates *AtMYC2* in both JA-dependent gene expression and inhibition of root growth. The results indicate that this novel MKK3–MPK6 cascade is part of an important JA signal transduction in *Arabidopsis*. Possible roles of the MKK3–MPK6 cascade in JA signaling are discussed.

Materials and Methods

Preparation of GST fusion proteins

Expression and affinity purification of glutathion S-transferase (GST) fusion proteins (Mizoguchi et al., 1993; Mizoguchi et al., 1998) were performed as follows. *E. coli* JM109 cells transformed with the GST fusion constracts were grown at 37℃ overnight and were sub-cultured until the OD_{600} reached 0.5. Expression of the GST-MPK fusion proteins was induced with 0.01 mM isopropyl- β -D-thiogalactopyranoside (IPTG). Cells were incubated at 16℃ overnight, then harvested by centrifugation at 5000*g* for 15 min at 4℃. The pellets were re-suspended in ice-cold 1 x PBS with 2 mM DTT, 2 μg ml^{-1} leupeptin, 2 μg ml^{-1} pepstatin A, 2 mM PMSF and 5 mg ml^{-1} lysozyme. The bacterial cells were disrupted by sonication. Triton X-100 was added to a final concentration of 1 %. The samples were gently inverted for 30 min at 4℃, and cell debris was removed by centrifugation at 12 000g for 10 min at 4℃. Beads of glutathion-Sepharose 4B (Amersham Pharmacia Biotech) were added to the supernatant and incubated with gentle inverting for 30 min at 4℃. The Sepharose beads were washed three times with 1 x PBS. The GST-MPK fusion proteins were eluted from the Sepharose beads three times using 10 mM-reduced glutathione in 50 mM Tris-HCl (pH 8.0). The eluted fractions were combined. Exchange of buffer with TTBS (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1 % v/v Tween 20) and protein concentration were simultaneously performed on a Microcon-30 column (Millipore).

Preparation of constitutive active form MAPKK and kinase inactive MAPK constructs

The constitutively active mutants of the MAPKKs were generated by QuickChange site-directed mutagenesis (Stratagene) and confirmed by sequencing.

The serine or threonine residues in activation loop domain [S/T] xxxxx [S/T] were replaced by acidic glutamate amino acids: MKK3DD (S235D T241D), MKK4DD (T224D S230D). The conserved lysine residue in the ATP-binding domains were replaced by the methionine and arginine amino acids MPK1-KN (K61M K62R), MPK3-KN (K67M K68R), MPK4-KN (K72M K73R), and MPK6-KN (K92M K93R), respectively.

The* in vitro *activation assay

The coding regions of MPK1-KN, MPK3-KN, MPK4-KN, MPK6-KN, MPK1, MPK3, MPK4, MPK6, MKK3, MKK3DD, MKK4, and MKK4DD were cloned into the pGEX4T-1 vector and expressed as glutathione S-transferase (GST) fusion proteins in JM109 *E.coli* cells (TOYOBO). Each GST-MPKs (1 μg) was incubated in 10 μl of kinase reaction buffer (50 mM Tris-HCl pH 7.5, 1 mM DTT, 10 mM $MgCl_2$, 10 mM $MnCl_2$, 50 μM ATP, and 0.037 MBq [γ -^{32}P]ATP (60 Ci mmol^{-1})) either without or with GST-MKKs or constitutive active GST-MKKs at 30℃ for 30 min. After adding 10 μl of kinase reaction containing 5 μg of myelin basic protein (MBP), the mixture was incubated at 30℃. Kinase reactions were stopped after 30 min by adding 4 μl 6xSDS sample buffer and heating for 4 min at 95℃. Reaction products were analyzed by SDS-PAGE, autoradiography, and Coomassie brilliant blue R250 staining.

Plant materials and treatments

The Columbia ecotype of *Arabidopsis thaliana* was used. The seeds of wild-type, *mkk2-2*, *mkk3-1*, *mpk6-4*, *mpk6-5*, *coi1*, *atmyc2-3*, and transgenic plants

were sterilized with 70 % ethanol and 0.5 % antiformin, and sown on GM agar plates containing 1 % sucrose. They were grown under continuous light at 22°C. Two-week-old seedlings of each transgenic plant were then transferred to 3 ml water for 24 h, at which point the water was replaced with 10 μM DEX or 50 μM JA solution. Samples were incubated for the indicated times. The MPK6 T-DNA null lines were obtained from the Kazusa DNA Research Institute (Kisarazu, Japan); the MKK3 (SALK_051970) and AtMYC2 (SALK_017005) T-DNA null lines were from the Arabidopsis Biological Resource Center (Columbus, OH); and the MKK2 T-DNA null line (FLAG_629G03) was obtained from the Institute National de la Recherche Agronomique (INRA) at the Jean-Pieere Bourgin Institute (Paris, France). The *mkk2-2* mutant was originally in Ws, and the F3 populations introgressed twice into Columbia. Lines that were putatively homozygous for T-DNA insertion were subjected to RT-PCR or Western blot analysis. The following gene-specific primers were used for RT-PCR analysis: *MKK2*, forward 5'-ATG AAG AAA GGT GGA TTC AG-3' and reverse 5'-CAC GGA GAA CGT ACC AGA CAG-3'; *MKK3*, forward 5'-CAG TTA TTC TTA TCC AGC TG-3' and reverse 5'-AAG CCA AGA TCT TTG AAA CTC GG-3'.

Dexamethasone and JA treatment

Two-week-old seedlings of each transgenic plant and vector control grown on the GM plate were transferred to stand for 24 h in 3 ml water. Then I added 5 μM DEX or 50 μM JA (f.c.) for indicated times.

Northern blot analysis

Northern blot analysis was performed as described (Yamaguchi-Shinozaki and Shinozaki, 1994). A coding region (nucleotide 1-1560) of *MKK3*, a coding region (nucleotide 1-1098) of *MKK4*, and a coding region (nucleotide 1-1185) of *MPK6* were used as probes for Northern blot analysis.

Microarray analysis

Total RNA was isolated with Trizol reagent (Invitrogen, Carlsbad, CA) and used for the preparation of Cy5- and Cy3-labeled cDNA probes. Microarray experiments were performed using the Agilent Arabidopsis 2 (*Arabidopsis* 22K) Oligo Microarray (Agilent Technologies, Palo Alto, CA). For each biological replicate, material from five plants was pooled to make a single sample for RNA purification. Two independent transgenic lines of *MKK3DD* and *MKK4DD* (*MKK3DD*#5 and *MKK4DD*#7 for Experiment 1; *MKK3DD*#3 and *MKK4DD*#2 for Experiment 2) were used for each experiment. All microarray experiments, including the data analysis, were performed according to the manufacturer's manual (http://www.chem.agilent.com/scripts/generic.asp?lpage=11617&indcol=Nandprodcol=Y). The reproducibility of microarray analysis was assayed by a dye swap in each experiment. On the basis of my empirical findings, expression of genes showing average signal intensity values of <500 to ~1000 in either the Cy3 or Cy5 channel of the control plants was not always detected reproducibly by RNA gel blot analysis. Thus, under my experimental conditions, genes showing a signal value <1000 in both Cy3 and Cy5 channels of the control plants were not considered for the analysis. I studied genes with *P* values <0.001. Feature extraction and image analysis software (version

A.6.1.1; Agilent Technologies) was used to locate and delineate every spot in the array and to integrate each spot's intensity, filtering, and normalization by the Lowess method. Gene clustering analysis was performed with Genespring 6.1 (Silicon Genetics, San Carlos, CA).

Quantitative RT-PCR analysis

Total RNA was treated with RNase-free DNase (Invitrogen). First-strand cDNA produced by the reverse transcription reaction was used as the subsequent template, amplified with real-time PCR master mix, and analyzed using the Gene Amp 7700 Sequence Detection System (Applied Biosystems, Foster City, CA). The relative expression levels in each coda sample were obtained by normalization to the *β-actin* gene. The following gene-specific primers for real-time PCR analysis were used: *β-actin2*, forward 5'-AGT GGT CGT ACA ACC GGT ATT GT-3' and reverse 5'-GAT GGC ATG AGG AAG AGA GAA AC-3'; *ACS6*, forward 5'-CCG ATG AAG AGT TTG TAG ACG AGT T-3' and reverse 5'-ATC TCA GCG TGC CTT GCA G-3'; *FRK1*, forward 5'-GAT TTC AAC AGT TGT CGC TGG AT-3' and reverse 5'-ACA TCA CTC TTT TCG TTC ATT TGG-3'; *AtLOX2*, forward 5'-TTG CAT CCT CAT TTC CGC TAC-3' and reverse 5'-CCT CCG TTG ACA AGA CTT TGG-3'; *PDF1.2*, forward 5'-TTT GCT GCT TTC GAC GCA C-3' and reverse 5'-CGC AAA CCC CTG ACC ATG-3'; *VSP2*, forward 5'-TCA GTG ACC GTT GGA AGT TGT G-3' and reverse 5'-GTT CGA ACC ATT AGG CTT CAA TAT G-3'; *AtMYC2*, forward 5'-TCA TAC GAC GGT TGC CAG AA-3' and reverse 5'-AGC AAC GTT TAC AAG CTT TGA TTG-3'.

Preparation of protein extracts

Frozen cultured cells were ground in liquid nitrogen, then thawed in extraction buffer (50 mM HEPES-KOH pH 7.5, 5 mM EDTA, 5 mM EGTA, 2 mM DTT, 25 mM NaF, 1 mM Na_3VO_4, 50 mM β-glycerophosphate, 20 % glycerol, 2 μg ml^{-1} leupeptin, 2 μg ml^{-1} pepstatin A, 2 mM PMSF). After centrifugation at 15 000g for 30 min at 4℃, supernatants were transferred into clean tubes, frozen in liquid nitrogen, and stored at -80℃. The protein content was determined by a turbidimetric procedure (Vera 1988).

Antibody production and immunoblot analysis

MPK1-, MPK4-, and MPK6-specific antibodies (MPK1NT, Ab4CT1 and Ab6NT1) were produced against synthetic peptides corresponding to the N-terminus of AtMPK1 (MAT LVD PPN GIR NEG C), the C-terminus of AtMPK4 (ELI YRE TVK FNP QDS V), and the N-terminus of AtMPK6 (MDG GSG QPA ADT EMT EAP GGF PGG FPA AAP S), respectively. The synthesized peptides were conjugated with keyhole limpet hemocyanin carrier. Polyclonal antisera were raised in rabbits and purified by affinity chromatography (Sawady Technology Inc, Tokyo, Japan). MPK3-specific antibody (MPK3NT) was provided from Dr. Scott C. Peck (University of Missouri-Columbia). For immunoblot analysis, 20 ng of each GST fusion protein and 20 μg of *Arabidopsis* leaf total protein were separated on a 10% polyacrylamide gel, and transferred to PVDF membrane by semidry electroblotting. After blocking for 1 h in TTBS buffer containing 5% non-fat dried milk (Yukijirushi, Sapporo, Japan) at room temperature, the membrane was incubated in TTBS buffer with MPK1NT (1/1000 didution), MPK3NT (1/10000), Ab4CT1 (1/5000) or Ab6NT1 (1/1000) for 1 h

at room temperature. After washing twice in TTBS buffer, the blots were incubated with a horseradish peroxidase-conjugated secondary antibody (Amersham, Buckinghamshire, UK), and the complexes were made visible by ECL (Enhanced Chemiluminescence) (Amersham) following the manufacturer's instructions.

Immunoprecipitaion

Crude extracts of cell cultures were adjusted to a protein concentration of 1 mg ml^{-1} and gently agitated in IP buffer (50 mM HEPES-KOH pH 7.5, 5 mM EDTA, 5 mM EGTA, 2 mM DTT, 25 mM NaF, 1 mM Na_3VO_4, 50 mM β-glycerophosphate, 20 % glycerol, 150 mM NaCl, 1 % Nonidet P-40) with 20 μl of a 50 % suspension of protein A-Sepharose CL-4B (Amersham Pharmacia Biotech) for 1 h at 4℃. For immunoprecipitation of MPK1NT, MPK3NT, Ab4CT, and Ab6NT, 500 μg, 100 μg, 500 μg, and 100μg of crude extracts were used, respectively. The mixture was centrifuged at 12 000*g* for 5 min at 4℃ and the supernatant was transferred to a new tube containing 10 μl, 1 μl, 10 μl, and 4 μl volume of each antibody. The mixture was gently agitated at 4℃ overnight. After adding 20 μl of the protein A beads, the mixture was agitated for 1 h at 4℃. The protein A beads were collected by centrifugation at 4000 *g* for 1 min, washed three times with 500 μl IP buffer, and then suspended in SDS sample buffer.

In-gel kinase assay

The in-gel kinase assay was performed as described previously (Zhang and Klessig, 1997; Romeis et al., 1999). In brief, 20 μg of protein per lane was subjected to electrophoresis on a 10 % polyacrylamide gel that contained SDS and 0.25 mg of

bovine brain myelin basic protein (MBP; Shigma-Aldrich) per ml. After electrophoresis, the gel was washed three times with washing buffer (25 mM Tris-HCl pH 7.5, 0.5 mM DTT, 0.1 mM Na_3VO_4, 5 mM NaF, 0.5 mg ml^{-1} BSA, 0.1 % Triton X-100) for 30 min at room temperature, followed by three washes with renaturation buffer (25 mM Tris-HCl pH 7.5, 1 mM DTT, 0.1 mM Na_3VO_4, 5 mM NaF) at 4℃ overnight. The gel was washed with reaction buffer (25 mM Tris-HCl pH 7.5, 2 mM EGTA, 12 mM $MgCl_2$, 1 mM DTT, 0.1 mM Na_3VO_4) for 30 min at room temperature and then incubated in 12.5 ml of reaction buffer with 250 nM ATP plus 1.85 MBq [γ -^{32}P] ATP (3 000 Ci $mmol^{-1}$) for 90 min at room temperature. The gel was washed five times with washing solution (5 % TCA, 1 % phyrophosphoric acid) and once with 5 % glycerol. The gel was dried on Whatman 3MM paper and exposed to RX-U film (Fuji Photo Film) or BAS2500 imaging plate (Fuji Photo Film).

Immunocomplex kinase assay

After immunoprecipitates were washed with 1 ml of reaction buffer without ATP (25 mM Tris–HCl pH 7.5, 2 mM EGTA, 12 mM $MgCl_2$, 1 mM DTT, 0.1 mM Na_3VO_4) (Zhang et al., 1998), kinase assays were performed in 20 μl of the same buffer containing 25 μM of ATP, 1 μ Ci of γ -^{32}P -ATP, and myelin basic protein (MBP) as a substrate at 30°C for 30 min. The reaction was stopped by the addition of sample buffer. After electrophoresis on a 12 % SDS-gel, the phosphorylated MBP was visualized by autoradiography.

ET production

Two-week-old seedlings of each transgenic plant and vector control grown

on the GM plate were transferred to stand for 24 h in 1 ml water in 7 ml vial container. Then I added 5 μM DEX or 50 μM JA (f.c.) for indicated times. The 1 ml air was collected from the vial and analyzed. The concentration of ethylene was determined with a gas chromatograph (GC-8A, SHIMADZU, Kyoto, Japan), equipped with an alumina column (Porapak Q 50/80; Shinwa, Kyoto, Japan) and a flame-ionization detector.

H_2O_2 detection by 3,3'-diaminobenzidine uptake method

Whole plant was dipped into the 1 mg/ml diaminobenzidine (DAB) solution, and incubated 8 h in dark room. After briefly rinse the samples in water, the leaves were clear by boiling in lactic acid/glycerol/EtOH (1:1:3) during 5 min in water bath. The samples was decolored with 2.5 g/ml of chloral hydrate, and replaced with 60% glycerol.

Quantitation of JA

Quantitation of JA and its methyl ester (MeJA) was performed as detailed previously (Seo et al., 1995), except that an HP6890 gas chromatograph fitted to a quadrupole mass spectrometer (Hewlett-Packard, Wilmington, DE) was used as the gas chromatography-mass spectrometry instrument.

Accession numbers

The microarray data were submitted in MIAME-compliant (minimum information about a microarray experiment) format to the ArrayExpress database (http://www.ebi.ac.uk/arrayexpress/) and have been assigned the accession number

E-MEXP-827. Sequence data from this article can be found in the GenBank/EMBL data libraries under the accession numbers as follows: ACS6, At4g11280; AtLOX2, At3g45140; AtMYC2, At1g32640; FRK1, At2g19190; MPK1, At1g10210; MPK2, At1g59580; MPK3, At3g45640; MPK4, At4g01370; MPK5, At4g11330; MPK6, At2g43790; MPK7, At2g18170; MKK2, At4g29810; MKK3, At5g40440; MKK4, At1g51660; PDF1.2, At5g44420; VSP2, At5g24770; β-actin, At3g18780.

Results

MKK3 specifically activates MPK6 *in vitro*

To explore which MAP kinase would be specifically activated by MKK3 *in vitro*, I established the *in vitro* activation assay system that analyzes the MAPK activation phosphorylated by MAPKK. Recently, MKK4 was reported to activate MPK3 and MPK6 in *Arabidopsis* protoplast system (Asai et al., 2002). Therefore, as a positive control, I first tested whether MKK4 activates MPK3 and MPK6 in this system.

I made MAPK or MAPKK proteins as a glutathione *S*-transferase (GST)-fusion protein in *Escherichia coli*. In general, MAPKKs require phosphorylation of the conserved serine (S) and/or threonine (T) residues in the kinase-activation loop for their activation (Marshall, 1994). Substitution of these S/T residues for aspartate (D) or glutamate (E) often resulted in constitutive activation of the MAPKKs and were used to activate target MAPK(s) both *in vitro* and *in vivo* (Cowley et al., 1994; Mansour et al., 1994). I produced a constitutively active form of MKK3 and MKK4 by substituting S/T for D/D (MKK3DD and MKK4DD; Figure 3), and tested whether or not active MAPKKs phosphorylated and activated the *Arabidopsis* MAPKs *in vitro*. I prepared GST-tagged WT and DD forms of MKKs, and various GST−MPK proteins, from *E. coli*. Wild-type MKK4 (MKK4WT) specifically activated MPK3 and MPK6 as reported (Figure 4A). In addition, the kinase activities in these two MAPKs were strongly enhanced by MKK4DD (Figure 4B), indicating that this *in vitro* activation assay system reflected the substrate specificity for MAPKKs.

An *in vitro* phosphorylation assay using kinase inactive MAPKs indicated that MKK3 directly phosphorylates MPK6 (Figure 5). Next, I used the wild-type and

constitutively active form of MKK3 (MKK3WT and MKK3DD) and explored the substrate MAPKs of MKK3 by using this system. MKK3WT specifically activated MPK6, but not other MAPKs including MPK3 (Figure 6A). However, MKK3DD-activated MPK6 showed more kinase activity than MKK3WT-activated MPK6 (Figure 6B). These results suggest that MKK3 possesses the ability to function as MAPKK, moreover MPK6 may be a downstream MAPK of MKK3 in *Arabidopsis*.

MKK3 actually activates MPK6 in *Arabidopsis*

Previously, it was reported that tobacco NtMEK2DD, which was fused to steroid-inducible promoter for overexpression (Aoyama and Chua, 1997), activated the endogenous MPK6/MPK3 in *Arabidopsis* as well as SIPK/WIPK in tobacco (Yang et al., 2001; Ren et al., 2002). I used this system to analyze MPK6 activation by MKK3 *in planta*. First, I treated DEX to *MKK4* transgenic plants for indicated times and their extracts were subjected to in-gel kinase assay. Four h after DEX treatment, the 46- and 44-kDa protein kinases were found to be specifically and strongly activated in *MKK4DD* plants (Figure 7A). Immunocomplex kinase (IP-kinase) assay using MPK6-specific antibody Ab6NT1 indicated that *MKK4DD* activated MPK6, as previously reported (Asai et al., 2002). Since the protein levels of MPK6 were equal during this experiment, these MPK6 activities were mainly due to *MKK4DD* overexpression (Figure 7C).

Next, I analyzed *MKK3* transgenic plants with DEX treatment. Four h after DEX application, the 46-kDa protein kinase was clearly activated in *MKK3DD* plants (Figure 8A). In contrast, *MKK3WT* only slightly activated the 46-kDa kinase. IP-kinase assay using MPK6-specific antibody indicated that this 46-kDa protein

kinase was MPK6 (Figure 8B). The protein levels of MPK6 were almost equal during this experiment (Figure 8C). The activation of MPK6 was similarly observed in the 2 independent lines of each transgene (data not shown).

To further confirm the identity of the activated kinases, I used specific antibodies raised against MPK1, MPK3, MPK4, and MPK6 for IP-kinase assays, using MBP as substrate. In *MKK3DD* plants, MPK6 was activated after 4 h of DEX treatment, but the MBP-kinase activity of MPK1, MPK3, and MPK4 was not affected (Figure 9). These results suggest that the activation of MPK6 by MKK3 is specific. In *MKK4DD* plants, MPK3 and MPK6 were activated as previously reported (Asai et al., 2002). These results indicate that the overexpression of constitutively active *MKK3DD* activates MPK6 in planta.

MKK3, MKK4, and *MPK6* are expressed in all tissues of *Arabidopsis*

To analyze the expression of the *MKK3*, *MKK4*, and *MPK6* genes in various *Arabidopsis* organs (root, stem, leaf, cauline leaf, flower, silique, and seed), I carried out Northern blot analyses (Figure 10). The *MKK3* mRNA was present in all the tissues examined. The levels of the *MKK4* mRNA detected were relatively higher in cauline leaves, flowers, and siliques than in stems and roots. These observations indicate that *MKK3* has different biological functions from those of *MKK4*. *MPK6* mRNA was detected equally in all the organs examined. These results also suggest that MKK3-MPK6 cascade functions in various tissues in *Arabidopsis* as well as *MKK4*.

MKK3DD does not activate ET biosynthesis

Recently, it was reported that MKK4-activated MPK6 phosphorylated one of

the major ET-synthesis enzyme ACS2/ACS6 and enhances the ET production (Liu and Zhang, 2004). Increase in ET biosynthesis occurs in plants under a wide variety of stresses. The two key steps in ET biosynthesis are the conversion of S-adenosyl-L-Met to 1-aminocyclopropane-1-carboxylic acid (ACC) and the oxidative cleavage of ACC to form ET (Hoffman et al., 1982). The enzymes catalyzing these two reactions are ACC synthase (ACS) and ACC oxidase (ACO), respectively. These enzymes encoded small gene families. In general, the basal activity level of ACS is very low in tissues that do not produce a significant amount of ET. Stress-induced ET production is associated with a rapid increase in cellular ACS activity. By contrast, ACO activity is constitutively present in most vegetative tissues. Therefore, ACS is the rate-limiting enzyme and the major regulatory step in stress-induced ethylene production (Bleecker and Kende, 2000; Wang et al., 2002). To confirm whether the biological function of MKK3-MPK6 cascade is similar to MKK4-MPK6 cascade through the MPK6 activation, I measured the ET levels in both *MKK4DD* and *MKK3DD* plants. I confirmed that the levels of ET accumulation became increasingly higher over time in DEX-applied *MKK4DD* plants (Figure 11). On the contrary, *MKK3DD* and control plants showed no increase of ET levels.

MKK3DD does not show the ET-dependent phenotype

In general, the accumulation of endogenous ET gas reads out the morphological phenotype of plants, named the triple response, when the plants overproduced ET gas are grown under the dark condition. It is suggested that triple response is consists of three distinct morphological changes in the shape of the seedling: inhibition of stem elongation, radical swelling of the stem, and absence of

normal geotropic response (diageotropism). Therefore, I also tested the ET-dependent morphological phenotypes of the *MKK3DD* and *MKK4DD* plants under dark condition. As positive control, I used constitutive triple response *constitutive triple response 1* (*ctr1*) mutant. Genetic studies showed that CTR1 was a negative regulator of ethylene responses in *Arabidopsis*. Its predicted protein sequence showed similarity to mammalian Raf-like kinase, and this kinase activity is essential for the function of CTR1 in ethylene signaling (Kieber et al., 1993; Huang et al., 2003). *ctr1-1* mutant indicates the constitutively activation of the ET signaling pathway, and shows the triple response grown under dark condition. *MKK4DD* plants and *ctr1-1* mutant exhibited strong triple response phenotype as expected (Figure 12). On the other hands, *MKK3DD*, *MKK3WT*, *MKK4WT*, and control plants did not cause the phenotype. These results indicate that MKK4-MPK6 cascade regulates ET biosynthesis but MKK3-MPK6 cascade does not, even though MKK3 activates MPK6 as well as MKK4 does.

Expression of *MKK3DD* does not lead to HR-like cell death in *Arabidopsis*

It has been suggested that the active mutant of *MKK4*, *MKK5*, and *NtMEK2*, the ortholog of *Arabidopsis MKK4*, activate two MAPKs, MPK3 and MPK6, and show HR-like cell death (Ren et al., 2002; Kim et al., 2003). Therefore, I compared the morphological phenotypes of the *MKK3DD* and *MKK4DD* plants treated with DEX on agar plates. These transgenic plants were grown under the MS plate for 10 days, then transfer to the MS plate containing 5 μM DEX. Under the absence of DEX, these transgenic plants grew with no morphological changes (Figure 13). *MKK4DD* plants exhibited strong HR-like cell death only when treated with DEX as expected.

MKK3DD, *MKK3WT*, *MKK4WT*, and control plants with or without DEX did not cause the HR-like cell death.

Expression of *MKK3DD* does not generate ROS

It has been reported that the activation of MKK4 cascade leads the generation of ROS and this causes HR-like cell death (Ren et al., 2002; Kim et al., 2003). Since ROS production has important roles in plant-pathogen interactions including HR-like cell death, I next used the DAB stain system whether H_2O_2 production was involved in this HR-like cell death phenotype. *MKK4DD* plants exhibited the reddish brown-colored precipitants of oxidized DAB that indicated the H_2O_2 production (Figure 14). I could not detect the brown stain in both *MKK3DD* and control plants. The DAB staining was the highest in tissues that were apparently healthy but were close to the collapsed tissues. However, after the whole leaf collapsed, it was no longer stainable with DAB. These data indicate that the DAB-detectable H_2O_2 accumulation immediately preceded the cell death. In addition, these results also suggest that MKK3-MPK6 and MKK4-MPK6 cascade have distinct roles in *Arabidopsis*.

MKK3 and MKK4 pathways regulates different genes expression

To identify genes acting downstream of MKK3 and representing a functional readout of its signaling, I used microarray analysis to compare the transcript levels of *MKK3DD* and *MKK4DD* plants. The steroid-inducible overexpression system shown in Figure 7 and 8 was applied to rule out any possibility of artificial effects, which are sometimes associated with constitutive overexpression systems. I analyzed the gene

expression profiles using the Agilent *Arabidopsis* 2 microarray, representing ~ 22,000 *Arabidopsis* genes (Agilent Technologies, Inc., Palo Alto, CA, USA). Because MPK6 activation became significant at 2–4 h after DEX treatment (Figure 7 and 8), I prepared total RNAs from seedlings treated with DEX for 4 h. The transcriptome profiles revealed that 56 and 100 genes increased in expression more than fivefold in *MKK3DD* and *MKK4DD* plants, respectively (Figure 15; Table 1 and 2). Among these genes, only 17 genes were co-regulated by both *MKK3DD* and *MKK4DD* (Table 3), suggesting that MKK3 and MKK4 have different roles in the regulation of gene expression in *Arabidopsis*.

I found distinct effects by *MKK3DD* and *MKK4DD* on the expression of the JA- and/or ET-regulated genes (Table 1 and 2). In the MKK4 signaling pathway, several disease resistance proteins, receptor-like kinases, ET biosynthesis genes, and WRKY transcription factors were found to be upregulated (Table 1). On the other hand, lipoxygenase, defensin proteins, and NAC domain proteins were upregulated in the MKK3 signaling pathway (Table 2).

I classified these upregulated genes according to their putative functions based on the classification of the Munich Information Center for Protein Sequence (MIPS) database to determine whether MKK3 and MKK4 pathway allocate gene expression patterns differently among classifications. Figure 16 shows a summary of the functional categorization of the transcripts increased in MKK3 and MKK4 pathway. The gene sets were subdivided into nine functional categories involved in unknown, metabolism, defense, transcription, cellular transport, cellular communication and signal transduction, protein fate, storage protein, and cellular biogenesis. In this result, the percentage of genes in each category is similar between two pathways.

MKK3DD affects mRNA levels of JA- and ET-regulated genes

MKK3DD and *MKK4DD* had distinct effects on the expression of JA- and/or ET-regulated genes. Next, I analyzed the time course dependent-expression about several genes upregulated in microarray analysis, to examine whether the gene expression pattern is different between MKK3 and MKK4 cascade.

Among them, I chose four genes, *AtLOX2*, *PDF1.2*, *ACS6*, and *FRK1*, as markers for a quantitative real-time PCR (QRT-PCR) analysis to further compare the possible roles of MKK3 and MKK4 in the JA- and ET-signaling pathways. *AtLOX2* encodes a chloroplast-targeted lipoxygenase that may be involved in the biosynthesis of JA from linolenic acid (Bell et al., 1995). The *PDF1.2* defensin gene expression is induced by pathogen infection (*Alternaria brassicicola*) and by treatments with JA and ET (Penninckx et al., 1998). *ACS6* gene expression is induced by stress (Wang et al., 2002), and ACS6 protein is phosphorylated by MPK6 (Liu and Zhang, 2004). *flg22-induced receptor-like kinaase 1* (*FRK1*) encodes an LRR receptor kinase and is induced by pathogens. Overexpression of either *MKK4DD* or *MKK5DD* increases *FRK1* expression (Asai et al., 2002). The *AtLOX2* and *PDF1.2* genes were upregulated as the activation of the MKK3 cascade was initiated (Figure 17), and the expression levels of these genes increased in *MKK4DD* plants after 12 h of DEX treatment. Expression levels of the *ACS6* and *FRK1* genes increased in *MKK4DD* plants, but not in *MKK3DD* plants.

MKK4DD plants showed the ET accumulation, triple response, and HR-like cell death phenotype when treated with DEX, but the *MKK3DD* plants did not in spite of activation MPK6 *in planta* (Figure 11-14). The HR-like cell death caused by ozone

is shown to be regulated by a balance between the JA and ET pathways (Tuominen et al., 2004). Considering the clear difference between *MKK3DD* and *MKK4DD* plants in terms of gene expression upon DEX treatment, I postulated that *MKK3DD* induction may modulate JA signaling in *Arabidopsis*.

Isolation of *MKK2, MKK3,* and *MPK6* knockout mutants in *Arabidopsis*

To analyze whether the MKK3-MPK6 cascade function in JA signaling in *Arabidopsis*, I obtained *MKK3* and *MPK6* knockout mutants. As a MAPKK control, I also obtained *MKK2* knockout mutant. To isolate *MPK6* knockout mutant, I used KAZUSA T-DNA knockout mutant lines and identified two mutant lines carrying a T-DNA insertion in *MPK6* (*mpk6-4* and *mpk6-5*; Figure 18A). The T-DNA element was inserted in the second intron (*mpk6-4*) and in the fifth exon (*mpk6-5*). I demonstrated that these lines were null mutants by immunoblotting using MPK6 specific antibody (Figure 18B). The *MKK3* knockout mutant (*mkk3-1*; Figure 18C) was isolated from SALK T-DNA lines. The T-DNA element was inserted in the seventh exon within the coding region of MKK3 in *mkk3-1*. PCR methods were used to confirm the homozygocity of *mkk3-1*, and RT-PCR analysis was used to confirm the loss of *MKK3* transcript in *mkk3-1* mutant, respectively (Figure 18D). The *MKK2* knockout mutant (*mkk2-2*; Figure 18E) was isolated from SAIL T-DNA lines. The T-DNA element was inserted in the second intron within the coding region of MKK2 in *mkk2-2*. PCR methods were used to confirm the homozygocity of *mkk2-2*, and RT-PCR analysis was used to confirm the loss of *MKK2* transcript in *mkk2-2* mutant, respectively (Figure 18F).

MPK6 is activated by JA

I first tested whether JA activated MPK6 in *Arabidopsis*. The *mpk6-4* mutant and the plants constitutively overexpressing MPK6 (*35S::MPK6-Myc*) were then treated with JA and analyzed to determine the activation of MPK6 by JA using an in-gel kinase assay (Figure 19, top panel). Wild-type plants showed JA-dependent activation of the 46-kDa protein kinase (MPK6), whereas the activation in the *mpk6-4* mutant specifically diminished. Interestingly, the activity of the 44-kDa protein kinase was higher in *mpk6-4* than in wild-type plants. The predicted molecular mass of the MPK6 protein with the Myc tags was around 65-kDa in *35S::MPK6-Myc*#15 plants, and this protein was recognized by Ab6NT1 (Figure 19, middle panel). JA activated not only endogenous MPK6, but also the fusion protein of MPK6-Myc.

***COI1* mutation affects the MPK6 activity by JA treatment**

In JA signaling, the several mutants have been isolated and displayed either reduced sensitivity to JA, methyl JA, and/or the JA analog coronatine or enhanced responses to JA. Among them, the coronatine insensitive mutant (*coi1*) was isolated for the mutant insensitive to growth inhibition by bacterial toxin coronatine. The *coi1* mutant is insensitive to growth inhibition by MeJA, male sterile, and fails to express JA-regulated genes (Benedetti et al., 1995; Xie et al., 1998; Thomma et al., 1999). Moreover, *COI1* is one of the most important key regulatory genes in JA signal pathway. *COI1* gene encodes a 66-kDa protein containing an N-terminal F-box motif and a leucine-rich repeat domain, identified as an F-box protein (Devoto et al., 2002; Xu et al., 2002). Therefore, to test how JA-insensitive *COI1* mutation affects the MPK6 activation, I analyzed the MPK6 activity using a JA-insensitive mutant *coi1*.

The *coi1* mutation specifically reduced JA-dependent MPK6 activity (Figure 20A and 20B). These data show that JA activates MPK6, and that the full MPK6 activation by JA requires *COI1*.

MPK4 is not activated by JA

Consider as the 44-kDa protein kinase was also activated by JA, I analyzed whether MPK3 and MPK4 are responsive to JA. It is well known that *Arabidopsis* MPK4 is one of the important signaling mediators in JA-signaling (Petersen et al., 2000; Brodersen et al., 2006). WIPK, a tobacco MPK3 homolog, is locally and systemically activated by wounding (Seo et al., 1999). Therefore, I examined whether JA actually activates MPK3 and MPK4 *in planta* by using IP-kinase assay, with MBP as a substrate. Consistent to Figure 19, MPK6 was reproducibly activated by JA with peaking at 5 to 10 min, but did not activate MPK4. No MPK4 activation by JA is consistent with the result by Brodersen et al. (2006). Interestingly, JA treatment rapidly activated MPK3 with peaking at 5 min. This result suggests that the 44-kDa protein kinase is most likely MPK3.

MKK3 plays a key role in the activation of MPK6 by JA

To test whether MKK3 is involved in the activation of MPK6 by JA, I analyzed MPK6 activation in wild-type, *mkk2-2*, *mkk3-1*, and MKK3 overexpressing plants (*35S::MKK3*). An in-gel kinase assay showed that the 46-kDa activity of MPK6 in wild-type plants was activated by JA (Figure 22A and 22B). As a MAPKK control, I performed the same experiment using the *mkk2-2* mutant. No significant difference in MPK6 activation by JA was observed between the *mkk2-2* mutant and wild-type plants.

Interestingly, MPK6 was only slightly activated in *mkk3-1* at 10 min, and the activation was much less than that in wild-type plants. In contrast, in the *35S::MKK3* plants, JA-dependent MPK6 activation was significantly higher than that in wild-type plants. Because JA contains a carboxyl group, I tested whether weak acid treatment (0.012% ethanol solution, pH 4.8) affected MPK6 activity in wild-type plants. Although I detected a slight increase in the 46-kDa kinase (MPK6) activity, the activation by this treatment was much weaker than that by JA. This shows that the MAPK activation by JA treatment did not occur in response to the low pH of the treatment.

To see MPK6 activity more directly in the different genotypes used in Figure 22, I performed IP-kinase assay of MPK6. This assay clearly demonstrated again that MPK6 is strongly activated by JA, and that the *35S::MKK3* plant and *mkk3-1* mutant resulted in enhanced and dropped JA-dependent MPK6 activity, respectively (Figure 23). These evidences confirm the results obtained in Figure 22 and also show that MKK3 is upstream of MPK6 in JA signaling. Since protein kinase activities of MPK6 was changed in *mkk3* and *35S::MKK3*, I compared the protein levels of MPK3, MPK4, and MPK6 in wild-type, *mkk2-2*, *mkk3-1*, *35S::MKK3*#13, and *mpk6-4* plants. Immunoblot analysis with specific MAPK antibodies showed that these protein levels remained constant with or without JA treatment in these mutants (Figure 23). These results indicate that the changes in MPK6 activity in different mutant backgrounds were not caused by variation in the protein levels, but rather by posttranslational modification of MPK6. These results also suggest that the loss-of-function and gain-of-function of *MKK3* mainly affected the activity of MPK6.

MKK3–MPK6 cascade affects JA-regulated root growth inhibition

Exogenously applied JA inhibits root growth in *Arabidopsis* (Staswick et al., 1992; Feys et al., 1994; Berger et al., 1996). Therefore, I investigated whether JA-dependent control of root growth is affected by loss-of-function mutants and transgenic overexpressors of *MKK3* and *MPK6* using the JA-signaling-related mutants *coi1* and *atmyc2-3* as controls. The root growth of *mkk3-1* and transgenic plants was similar to that of wild-type plants without JA (Figure 24A and 24B). The *mpk6* mutant plants had accelerated root growth compared to wild-type plants. Increasing the concentration of JA caused severe root growth inhibition in two independent alleles of *mpk6* mutants. JA-mediated root growth inhibition was also observed in *mkk3-1* compared to wild-type plants. On the other hand, the root growth of the *mkk2-2* mutant was inhibited to a similar level as the wild-type. In contrast, two independent lines of each *35S::MKK3* and *35S::MPK6-Myc* plants showed less sensitivity to JA, at a level comparable to that of *atmyc2-3*. The *coi1* mutant showed a strong insensitive phenotype to JA, as previously reported (Feys et al., 1994).

I obtained *MKK3* knockout mutant as single allele. Therefore, I also tested whether the root growth inhibition phenotype in *mkk3-1* mutant depends on the *MKK3* function using the *MKK3* complementation transgenic plants by *35S::MKK3* in *mkk3-1* background. The plant transformation vector (pBE2113Not-*MKK3*) was used to transform the *mkk3-1* mutant plants. Three independent *MKK3*/*mkk3-1* plants (line 1, 2, and 5) were isolated by RT-PCR, respectively (Figure 25A). Without exogenous application of JA, root growth of these mutants was similar to that of wild-type plants (Figure 25B and 25C). The *mkk3-1* plants showed higher sensitivity to JA than that of wild-type plants as shown in Figure 24. This phenotype was rescued by *35S::MKK3*,

indicating that the JA-sensitive phenotype of the *mkk3-1* plants is caused by the loss-of-function of *MKK3*.

The *mkk2* alleles were already described to have less sensitivity to salt stress (Teige et al., 2004). Compared to wild-type plants, *mkk2-2* mutant also showed decreased sensitivity to 100 mM NaCl, but *mkk3-1* did not (Figure 26A and 26B). These results support my hypothesis that MKK3–MPK6 cascade specifically regulates JA signaling, but not salinity stress response.

MKK3-MPK6 cascade does not regulate JA biosynthesis

I confirmed that one of the JA biosynthesis genes, *AtLOX2*, was highly up-regulated in steroid-inducible *MKK3DD* plants (Figure 17; Table 1). Moreover, the expression level of the *12-oxo-phytodienoic acid reductase 3* (*OPR3*) in *MKK3DD* plants was 3-fold higher than that in control plants (http://www.ebi.ac.uk/arrayexpress/). A previous study suggested that a positive feedback regulatory system might control JA biosynthesis (Sasaki et al., 2001). To analyze whether MKK3 cascade regulates JA biosynthesis, I measured JA production in *MKK3DD* plants (Table 4). But I did not find any changes in JA levels in these plants. Based on this result, the stimulation of JA biosynthesis by MKK3 is unlikely, and MKK3 is most likely involved in JA signaling.

MKK3–MPK6 cascade affects the genes expression controlled by JA

PDF1.2 and *VSP2* genes are commonly used markers for JA signaling. *PDF1.2* is regulated by the JA/ET pathway (Penninckx et al., 1998), and *VSP2* is regulated by the JA pathway (Berger et al., 1996), but cross talk occurs between these

two pathways, as revealed by their antagonistic regulation (Boter et al., 2004; Lorenzo et al., 2004). Microarray analysis and QRT-PCR showed that *PDF1.2* was upregulated in response to the *MKK3DD* induction (Figure 17; Table 2). To elucidate the role of the MKK3–MPK6 cascade in the cross talk between the JA and JA/ET pathways, I investigated mRNA levels of *PDF1.2* and *VSP2* genes in both loss-of-function mutants and transgenic overexpressors of *MKK3* and *MPK6* (Figure 27). In the absence of JA, the basal expression of *PDF1.2* in the *35S::MPK6-Myc* plant was higher than that in wild-type plants. JA significantly increased the level of the *PDF1.2* transcript in the *35S::MKK3* and *35S::MPK6-Myc* plants. The expression level of *PDF1.2* in *atmyc2-3* was also higher than that in wild-type plants, as previously reported (Boter et al., 2004; Lorenzo et al., 2004). In contrast, all the *mkk3-1*, *mpk6-4*, and *mpk6-5* mutants showed a significantly lower JA-dependent *PDF1.2* expression and an increased JA-induced *VSP2* expression. Consistently, *VSP2* expression levels in both *35S::MKK3* and *35S::MPK6-Myc* plants were suppressed to an extent similar to that of *atmyc2-3*. In the *coi1* mutant, JA-dependent expression of the *PDF1.2* and *VSP2* genes was suppressed largely as previously reported (Devoto and Turner, 2003). The *mkk2-2* mutant did not show a significant change in the expression level of *PDF1.2* and *VSP2* relative to wild-type plants. These results suggest that the MKK3–MPK6 cascade positively and negatively regulates gene expression of the *PDF1.2* and *VSP2*, respectively.

MKK3-MPK6 cascade does not regulate positively the JA/ET pathway through ET biosynthesis and *ERF1* expression

Resent study revealed that the cooperation or antagonism between JA and ET

is regulated by *ethylene response factor 1* (*ERF1*) and *AtMYC2*. ERF1 functions as one of the most important activator of the JA/ET-dependent pathway and represses the expression of JA specific pathway mediated genes (Lorenzo et al., 2003). On the other hand, AtMYC2 regulates the JA specific pathway mediated genes expression and represses JA/ET-dependent pathway mediated genes expression (Boter et al., 2004; Lorenzo et al., 2004). To analyze whether the increased *PDF1.2* expression by the MKK3–MPK6 cascade is mediated through the ET pathway, I measured ET production in both loss-of-function mutants and transgenic overexpressors of *MKK3* and *MPK6* treated with or without JA (Figure 28). The accumulation of ET production in *mkk3-1*, *mpk6-4*, and *35S::MKK3#*13 plants without JA was similar to that in wild-type plants, and these differences were not statistically significant (*mkk3-1*, P = 0.397479; *mpk6-4*, P = 0.329222; *35S::MKK3#*13, P = 0.4300025). Only *35S::MPK6-Myc#*15 had a higher amount of ET than that in wild-type plants, and this difference was statistically significant (*35S::MPK6-Myc#*15, P = 0.04186 < 0.05). Basal ET levels increased in *35S::MPK6-Myc#*15, possibly due to the slight upregulation of ACS, which is a substrate of MPK6 (Liu and Zhang, 2004). In a similar way, JA did not significantly affect the ET levels in wild-type, *mkk3-1*, *mpk6-4*, 35S::*MKK3#*13, and 35S::*MPK6-Myc#*15 plants (Col., P = 0.871529; *mkk3-1*, P = 0.875416; *mpk6-4*, P = 0.617229; *35S::MKK3#*13, P = 0.571416; *35S::MPK-Myc#*15, P = 0.461129).

Next, I analyzed whether the MKK3-MPK6 cascade-induced expression of *PDF1.2* mediates the *ERF1* expression using *mkk3* and *mpk6* mutants. In *mkk3* and *mpk6* mutants, the *ERF1* expression level was similar to wild-type plants (Figure 29). On the contrary, the *PDF1.2* expression was reduced in these mutants (Figure 27).

These results indicate that the *PDF1.2* expression downstream of JA-dependent MKK3-MPK6 cascade did not correlate to the *ERF1* expression.

PDF1.2 expression regulated by JA-activated MKK3-MPK6 cascade also requires endogenous ET signaling

In response to different pathogens, jasmonate and ethylene cooperate to induce defense genes such as *PDF1.2* and basic *PR* protein. Analyzing *ethylene insensitive* mutant (*ein2*) and *coi1* mutant, *PDF1.2* expression requires concomitant triggering of the JA and ET pathways; i.e., if either pathway is not activated, expression cannot occur (Penninckx et al., 1998). To confirm this concomitant regulation, I treated these mutants with 50 μM JA or 50 μM ACC that is the precursor of ET, and measured the expression level of *PDF1.2* and *VSP2* using QRT-PCR. The *Arabidopsis EIN2* gene is a central component of the ET signaling pathway. The amino-terminal integral membrane domain of *EIN2* shows similarity to the disease-related Nramp family of metal-ion transporters (Alonso et al., 1999). The well known component of the ethylene pathway that works in downstream of *EIN2* is *EIN3*, a member of a plant-specific transcription factor family (Chao et al., 1997). In addition, five additional *EIN3-like* (*EIL*) genes have been identified in the *Arabidopsis* genome (The *Arabidopsis* Genome Initiative, 2000). ET-induced *PDF1.2* expressions were strongly inhibited by *ein2* and *ein3* mutants (Figure 30). In addition, JA-induced *PDF1.2* expressions were strongly inhibited by *ein2* only. On the contrary, *Arabidopsis* has *EIN3* homologs, *EIL1-3*, that have redundant roles with the *EIN3*; therefore, the *ein3* mutation only partially suppressed the induction of the *PDF1.2* gene by JA treatment.

I also tested how ET-insensitive *ein2* and *ein3* mutations affect JA-dependent expression of *PDF1.2* by *35S::MKK3*. In wild-type plants, *PDF1.2* gene expression was induced by JA and ACC, as previously reported (Penninckx et al., 1998). *PDF1.2* expression in the *35S::MKK3* plants treated with JA was higher than that in wild-type plants treated with JA. To test whether ET signaling was required for the JA-dependent induction of *PDF1.2* expression, two ET-insensitive mutants, *ein2* and *ein3*, were used to generate double mutants of *35S::MKK3/ein2-5* and *35S::MKK3/ein3-1*. The *ein2* mutation completely suppressed the JA-dependent induction of *PDF1.2* expression in the *35S::MKK3* genetic background. The *ein3* mutation also reduced *PDF1.2* expression in *35S::MKK3*, but suppression by *ein3* was weaker than that by *ein2*. Similar effects by *ein2* and *ein3* on the JA-dependent induction of *PDF1.2* expression were observed in wild-type plants. This may be reasonable, because *EIN3* and its close homologs, *EIL1-3*, have redundant functions in ET signaling in *Arabidopsis* and the ET-insensitive phenotype of *ein3* is much weaker than that of *ein2* (Stepanova and Alonso, 2005). These results indicate that ET signaling was required for the JA-dependent induction of *PDF1.2* expression.

To test whether the *ein* mutations affect JA-dependent activation of MPK6, I examined the MPK6 activity in *ein2-5*, *ein3-1*, and wild-type plants. The MPK6 activity in *ein2-5* and *ein3-1* mutants was almost the same as that in wild-type plants (Figure 32). Therefore, the increased levels of *PDF1.2* observed in the MKK3–MPK6 cascade may be mainly attributable to JA signaling and be concomitantly upregulated.

MKK3-MPK6 cascade plays a key role in the JA-dependent negative regulation of *AtMYC2*

AtMYC2 encodes a bHLH transcription factor and plays a predominant role in the JA pathway (Boter et al., 2004; Lorenzo et al., 2004). To investigate whether the *AtMYC2* could function as a downstream factor of the MKK3–MPK6 cascade in JA signaling, the JA-dependent expression of *AtMYC2* was tested (Figure 33). *AtMYC2* expression markedly reduced in the independent lines of each *35S::MKK3* and *35S::MPK6-Myc* plants, increased in the JA-treated *mkk3-1*, *mpk6-4*, and *mpk6-5* mutants, and was similar to that of wild-type plants in the *mkk2-2* mutants. This result was unexpected, because JA has been thought to regulate the *AtMYC2* expression positively. My results suggest that the JA activates the MKK3–MPK6 cascade and the activated kinases may negatively regulate the *AtMYC2* expression. This is the first demonstration of a possible negative regulation of the *AtMYC2* expression by JA. In addition, these results are consistent with the data on the JA-dependent root growth inhibition (Figure 24).

Next, I analyzed the expression levels of *PDF1.2* and *VSP2* using an *mkk3-1*/*atmyc2-3* double mutant (Figure 34). *PDF1.2* expression was repressed in the *mkk3-1* mutant (Figure 27 and 34). Interestingly, the suppressed *PDF1.2* expression in the *mkk3-1* was reversed back to a level similar to that of the *atmyc2-3* single mutant by introducing the *atmyc2-3* mutation to the *mkk3-1* (Figure 34, left panel). The induction of *VSP2* expression was consistently repressed in the *mkk3-1*/*atmyc2-3* mutant as well as in the *atmyc2-3* mutant (Figure 34, right panel). These results strongly suggest that *AtMYC2* is genetically downstream of *MKK3*.

Along this line, I further examined whether the JA-dependent root growth

sensitivity of *mkk3-1* is affected by *atmyc2-3* mutation (Figure 35A and 35B). Without exogenous application of JA, root growth of these mutants was similar to that of wild-type plants. In contrast, *mkk3-1*/*atmyc2-3* showed less sensitivity to JA, with a level comparable to that of *atmyc2-3*. These results indicate that i) JA regulates the *AtMYC2* expression both positively and negatively in two independent pathways, ii) *AtMYC2* has a role in downstream of the MKK3–MPK6 cascade, and iii) the MKK3–MPK6 cascade plays a key role in the JA-dependent negative regulation of the *AtMYC2*.

Discussion

Biochemical analysis of MAPKK and MAPK suggested that MKK3 as well as MKK4 can phosphorylate and activate MPK6. This novel cascade was also supported *in planta* using transgenic plant.

Loss-of-function mutants of *MKK3* and *MPK6*, and transgenic plants overexpressing *MKK3* and *MPK6*, were used to study the roles of a novel MAPK cascade, MKK3–MPK6, in *Arabidopsis*. Analyzing these mutants in combination with *coi1* and *atmyc2-3* revealed that the MKK3–MPK6 cascade plays a key role in JA signaling of *Arabidopsis*. This is discussed in further detail in the following sections.

An *in vitro* and *in planta* activation of MAPKs by MKK3

To identify the interactions among MAPKKs, and MAPKs in *Arabidopsis*, researches had taken two approaches: Y2H analysis on pairwise protein–protein interactions, and a functional complementation of yeast mutants by cotransformation of MEKK/MAPKK or MAPKK/MAPK. The first potential MAPK cascade in plants, MEKK1–MKK1/MKK2–MPK4 (Ichimura et al., 1998; Mizoguchi et al., 1998), was identified using these approaches. Since then, several other MAPK cascades have been identified using Y2H, functional complementation of yeast mutants by coexpression of the kinases, and the transient coexpression assay of those kinases using *Arabidopsis* mesophyll cell protoplasts. These include MKK4/MKK5–MPK3/MPK6 and MKK2–MPK6 (Asai et al., 2002; Ren et al., 2002; Teige et al., 2004). Here, I used an *in vitro* activation assay to identify novel functional interactions between MAPKKs and MAPKs. I confirmed MPK6 and MPK3 as target proteins of MKK4 in this system (Figure 4), as reported previously, and identified MPK6 as a possible downstream target of one of the least-characterized MAPKKs, MKK3. In addition, I showed that

MPK1 and MPK2 are slightly phosphorylated by MKK3 *in vitro* (Figure 6). Considering that MKK3 interacts with MPK1 in the Y2H (Ichimura et al., 1998), and that MPK1 and MPK2 are closely related to group C MAPKs (Ichimura et al., 2002), a functional link may exist between MKK3 and MPK1/MPK2 in *Arabidopsis*. Alternatively, MPK1 may not be an *in vivo* target of MKK3, because I did not detect the activation of MPK1 by MKK3DD in my experiment using an IP-kinase assay (Figure 9). Although MPK1 activity was not affected by MKK3DD under the conditions during the time course I tested, I cannot rule out the possibility that MPK1 may be activated transiently or in specific organs or tissues by MKK3. MPK6 did not interact directly with MKK3 in an Y2H analysis (Mizoguchi and Shinozaki, unpublished data). It is necessary to test whether MPK6 interacts with MKK3 *in vivo*.

To determine the specific roles of the MAPKKs in *Arabidopsis* plants, I used a conditional overexpression system to identify immediate molecular responses *in planta*. Previous works have reported that overexpression of the constitutively active form of MAPKKs driven by a DEX-inducible promoter can mimic the activation of its MAPK cascade in both tobacco and *Arabidopsis* (Yang et al., 2001; Ren et al., 2002). Using this system, I confirmed that the novel MKK3–MPK6 cascade identified by the *in vitro* activation assay really functions in *Arabidopsis* (Figure 8). Moreover, I found that constitutively active MKK3DD activates MPK6 more strongly in plant cells than that observed in the *in vitro* assay. This difference may derive from its structural effects, because MKK3 has a longer extra-kinase region on its C-terminus than other MAPKKs. The MKK3 protein produced in *E. coli* cells might not function properly, and may require some modification of the C-terminal region to achieve full activation. Domain analysis of the C-terminal region should reveal how it affects MKK3

activation.

Identification of the protein kinase cascade regulated by the JA pathway

Pharmacological studies using protein kinase or protein phosphatase inhibitors have revealed the important roles of protein phosphorylation and dephosphorylation in JA signaling (Leon et al., 2001). Treatment of *Arabidopsis* seedlings with the protein kinase inhibitor staurosporin stimulated the expression of the wound-regulated marker genes *VSP* and *JR1* in the absence of JA. In contrast, the expression of these JA-dependent genes was repressed by the protein phosphatase inhibitor okadaic acid. These results indicate that the JA pathway is negatively regulated by protein kinase cascades. In addition, several reports have suggested that MAPKs are involved in JA signaling (Seo et al., 1999; Petersen et al., 2000; Gomi et al., 2005). However, whether JA really activates MAPKs in plants was previously unknown. Here, I demonstrated JA-dependent MPK6 activity in *Arabidopsis* plants (Figure 19 and 21). This is the first evidence that MPK6 is activated by JA in addition to various kinds of abiotic and biotic stresses (Nakagami et al., 2005). *Arabidopsis* has ten MAPKKs classified into four groups (A–D) (Ichimura et al., 2002). MKK3 belongs to group B, and its biological function and substrate MAPK remains to be elucidated. The present study clearly showed that JA-induced MPK6 activation is mainly dependent on MKK3, because MPK6 activation by JA is strongly repressed in *mkk3-1*, but enhanced in *35S::MKK3* plants (Figure 22 and 23). Possibly, other MAPKKs may slightly activate MPK6 in *mkk3-1* mutants since the *mkk3* mutation did not completely diminish MPK6 activity. In addition, the *coi1* mutation also partially repressed JA-dependent MPK6 activation (Figure 20), strongly suggesting a crucial

function of the MKK3–MPK6 cascade in JA signaling. However, an apparent inconsistency exists between the degree of JA-insensitivity to root growth and partial suppression of JA-dependent MPK6 activation in the *coi1* mutant. Mechanical stress during the experiment could result in MPK6 activation, and may explain this inconsistency.

In addition to MPK6, 44-kDa protein kinase was also activated by JA (Figure 19 and 20). My data indicated that one of the candidates of the 44-kDa protein is MPK3 (Figure 21). In *mpk6* mutant, the activity of MPK3 increased higher than that in wild-type plants (Figure 19). This suggests that both MPK6 and MPK3 may function redundantly in JA signaling. Moreover, in *coi1* mutant, MPK3 activity did not changed, but MPK6 activity reduced (Figure 20). Therefore, each biological pathway of these proteins may function independently in COI1-related JA signaling. MKK3 activates MPK6, but not MPK3 *in planta* as well as *in vitro* (Figure 6 and 9). Although I have no evidence that MPK3 is a substrate of MKK3, this predicts presence of another MAPK cascade leading to MPK3 activation. Role of MPK3 in JA signaling needs to be determined. It would be very interesting to identify MAPKK required for MPK3 activation by JA and to elucidate a role of JA response of them using knockout mutants and gain-of-function plants.

Previous reports indicate that *Arabidopsis* MPK4 regulates *PDF1.2* expression through EDS1/PAD4 downstream or independently of ERF1 (Petersen et al., 2000; Andreasson et al., 2005). Therefore, I also tested whether MPK4 is responsive to JA; however I did not see any MPK4 activation (Figure 21). Considering MKK3 did not activate MPK4 *in planta* as well as *in vitro* (Figure 6 and 9), I think MPK4 is not an immediate target of MKK3. In *mpk4*, ET-induced *PDF1.2* induction was blocked

(Brodersen et al., 2006). By contrast, *35S::MKK3* plant showed almost wild-type level of ACC-dependent *PDF1.2* induction, not like the case of JA-dependent *PDF1.2* induction (Figure 31). Since the MKK3-MPK6 cascade functions upstream of *AtMYC2*, probably not in the upstream of *ERF1*, I think that MKK3-MPK6 pathway regulates *PDF1.2* through AtMYC2 function, but MPK4-EDS1/PAD4 pathway does downstream or independently of ERF1.

Function of the MKK3–MPK6 cascade in the cross talk between the JA and JA/ET pathways in *Arabidopsis*

I demonstrated that the novel MKK3–MPK6 cascade plays an important role in JA-controlled root growth and JA- and JA/ET-dependent gene expression (Figures 24 and 27). Recent studies revealed that the transcription of ET- and JA-induced genes is mainly regulated by two transcription factors, ERF1 and AtMYC2, respectively. ERF1 activates JA/ET-dependent gene expression, but represses JA-dependent gene expression (Lorenzo et al., 2003). In contrast, AtMYC2 promotes JA-dependent gene expression and represses JA/ET-dependent genes (Boter et al., 2004; Lorenzo et al., 2004). In Figure 36, I summarized a model of cross talk in JA and ET pathways and MAPK cascades. In this study, I demonstrated that the MKK3–MPK6 cascade positively regulates the expression of *PDF1.2* (Figure 27) without affecting *ERF1* expression (Figure 29). Moreover, the JA-activated MKK3–MPK6 cascade did not accumulate ET (Figure 28), indicating that the MKK3-activated MPK6 does not enhance ET biosynthesis, whereas the MKK4–MPK6 cascade does. Based on these results, I propose a model that the MKK3–MPK6 cascade may independently mediate the expression of *PDF1.2* without affecting ET synthesis. However, as previously

shown (Penninckx et al., 1998), *35S::MKK3/ein2* and *35S::MKK3/ein3* plants further demonstrated that the expression of *PDF1.2* regulated by the JA-activated MKK3–MPK6 cascade requires endogenous ET signaling (Figure 31 and 32). An alternative but still possible explanation for this is that the MKK3–MPK6 cascade might affect the ERF activity at a posttranslational level. A rice kinase, BWMK1, belongs to the group D of the MAPK family and is shown to be activated by various stresses and hormones including JA (Cheong et al., 2003). BWMK1 interacts with a rice homolog of ERFs.

I also found that the MKK3-MPK6 pathway negatively regulate JA-dependent *AtMYC2* expression. Because of key roles of AtMYC in both root growth inhibition and gene expression by JA (Boter et al., 2004; Lorenzo et al., 2004), phenotypes of *mkk3* and *mpk6* mutants as well as *35S::MKK3* and *35S::MPK6* can be explained by the expression level of *AtMYC2*. I note that the *AtMYC2* expression by JA is still increasing in both 35S::*MKK3* and 35S::*MPK6* background (Figure 33). In addition, simple overexpression of *JAMYC2* and *JAMYC10*, potato *AtMYC2* homologs, is not sufficient to induce JA-inducible genes (Boter et al., 2004; Lorenzo et al., 2004). These evidences suggest the presence of unidentified JA-induced positive regulation for *AtMYC2*, ant it's possibly competing with the MKK3-MPK6 pathway. Genetic analysis by producing *mkk3-1*/*atmyc2-3* double mutant showed that the MKK3–MPK6 cascade is epistatic to *AtMYC2* (Figure 34 and 35). In summary, I propose that the MKK3–MPK6 cascade negatively regulates JA-induced gene expression of *AtMYC2* upstream of the JA pathway, and that *AtMYC2* expression is controlled by a balance of the positive and negative pathways (Figure 36). This is the first demonstration of the negative regulation of the *AtMYC2* expression by JA.

Specificity of MAPK cascade in *Arabidopsis*

In *Saccharomyces cerevisiae*, high extracellular osmolarity treatment induces the Sln1–Ypd1–Ssk1 two-component osmosensor to activate a MAPK cascade composed of Ssk2 and Ssk22 (MAPKKKs), Pbs2 (MAPKK), and Hog1 (MAPK). A second osmosensor, Sho1, also activates Pbs2 and Hog1, but does so through Ste11 (MAPKKK). The MAPKK Pbs2, thought to serve as a scaffold protein, binds to the Sho1 osmosensor, the MAPKKK Ste11, and the MAPK Hog1. However, under mating signaling, Ste11, Ste7 (MAPKK), and Fus3/Kss1 (MAPKs) compose the pheromone-responsive MAPK cascade coordinated by the scaffold protein Ste5 (Posas and Saito, 1997). Thus, Ste11 is activated by two distinct signals, the high extracellular osmolarity and the mating pheromone, but regulates different signaling pathways through the Pbs2–Hog1 and the Ste7–Fus3/Kss1, respectively. Scaffold proteins such as Pbs2 and Ste5 appear to play important roles in selecting upstream and downstream factors of Ste11.

In *Arabidopsis*, four distinct MAPKKs activate the MPK6 and regulate different downstream responses. Therefore, distinct scaffold proteins may play a key role in each signaling pathway. Protein complexes that include the MKK3–MPK6, MKK4/MKK5–MPK6, and MKK2–MPK6 cascades need to be identified to understand both the upstream and downstream factors of the MAPK cascades (Figure 36).

Conclusion

In the present study, I showed that the MKK3–MPK6 and MKK4–MPK6 cascades regulate different signaling cascades, as revealed by both morphological and physiological phenotypes and gene expression profiles using steroid-inducible constitutively active form MAPKK in transgenic plants. I showed that the MKK3-MPK6 cascade functions in JA responsive signaling pathways in root growth and the expression of *AtMYC2*. I also demonstrated JA-dependent activation of MPK6 is controlled by MKK3 using *mkk3* mutant and *35S::MKK3* plants. In addition, MKK2–MPK6 was shown not to affect JA signaling on the basis of the JA-dependent MPK6 activation, gene expression, and root growth assay.

In summary, the present study identified a novel MAPK cascade, MKK3–MPK6, in *Arabidopsis*. Four MAPKKs, namely MKK2, MKK4, MKK5, and MKK3, activate MPK6 in *Arabidopsis* (Figure 36). However, the biological functions of the three cascades seem to be different. Presumably, MKK2, MKK4, MKK5, and MKK3 interact with MPK6 to constitute different MAP kinase complexes *in planta* to transduce different signals and cross talk with each other. I showed that the MKK3–MPK6 cascade is mainly involved in JA signaling. This cascade negatively regulates *AtMYC2* function and JA-dependent root growth sensitivity. In contrast, the MKK4/MKK5–MPK6 and MKK2–MPK6 cascades are involved in ET signaling and cold/salt stress signaling, respectively (Kim et al., 2003; Liu and Zhang, 2004; Teige et al., 2004).

Acknowledgements

I wish to express my sincere appreciation to Prof. K. Shinozaki (RIKEN Plant Science Center), Prof. H. Kamada, Prof. S. Satoh, and Prof. S. Sakai of the University of Tsukuba for their invaluable suggestions and encouragement throughout my research and preparation of this thesis.

I am also indebted to Dr. R. Yoshida and Dr. T. Mizoguchi of the University of Tsukuba, Dr. K. Ichimura of RIKEN PSC (Plant Science Center), Dr. K. Yamaguchi-Shinozaki of JIRCAS (Japan International Research Center for Agricultural Sciences, Ministry of Agriculture, Forestry, and Fisheries) for their invaluable suggestions, advices and constructive criticisms of my research.

I am also grateful to Dr. K. Maruyama of JIRCAS, Dr. S. Seo of NIAS (National Institute of Agrobiological Sciences) for their technical advances and helpful suggestions.

Special thanks must be according to all members of my laboratory and plant physiology group of Tsukuba University for useful discussion of my research.

References

Alonso, J.M., Hirayama, T., Roman, G., Nourizadeh, S., and Ecker, J.R. (1999). EIN2, a bifunctional transducer of ethylene and stress responses in Arabidopsis. Science **284,** 2148-2152.

Andreasson, E., Jenkins, T., Brodersen, P., Thorgrimsen, S., Petersen, N.H., Zhu, S., Qiu, J.L., Micheelsen, P., Rocher, A., Petersen, M., Newman, M.A., Bjorn N.H., Hirt, H., Somssich, I., Mattsson, O., and Mundy, J. (2005). The MAP kinase substrate MKS1 is a regulator of plant defense responses. EMBO J **24,** 2579-2589.

Aoyama, T., and Chua, N.H. (1997). A glucocorticoid-mediated transcriptional induction system in transgenic plants. Plant J **11,** 605-612.

Asai, T., Tena, G., Plotnikova, J., Willmann, M.R., Chiu, W.L., Gomez-Gomez, L., Boller, T., Ausubel, F.M., and Sheen, J. (2002). MAP kinase signalling cascade in Arabidopsis innate immunity. Nature **415,** 977-983.

Bell, E., Creelman, R.A., and Mullet, J.E. (1995). A chloroplast lipoxygenase is required for wound-induced jasmonic acid accumulation in Arabidopsis. Proc Natl Acad Sci USA **92,** 8675-8679.

Benedetti, C.E., Xie, D., and Turner, J.G. (1995). Coi1-dependent expression of an Arabidopsis vegetative storage protein in flowers and siliques and in response to coronatine or methyl jasmonate. Plant Physiol **109,** 567-572.

Berger, S. (2002). Jasmonate-related mutants of Arabidopsis as tools for studying stress signaling. Planta **214,** 497-504.

Berger, S., Bell, E., and Mullet, J.E. (1996). Two methyl jasmonate-insensitive mutants show altered expression of AtVsp in response to methyl jasmonate and wounding. Plant Physiol **111,** 525-531.

Berrocal-Lobo, M., Molina, A., and Solano, R. (2002). Constitutive expression of ETHYLENE-RESPONSE-FACTOR1 in Arabidopsis confers resistance to several necrotrophic fungi. Plant J **29,** 23-32.

Bleecker, A.B., and Kende, H. (2000). Ethylene: a gaseous signal molecule in plants. Annu Rev Cell Dev Biol **16,** 1-18.

Boter, M., Ruiz-Rivero, O., Abdeen, A., and Prat, S. (2004). Conserved MYC transcription factors play a key role in jasmonate signaling both in tomato and Arabidopsis. Genes Dev **18,** 1577-1591.

Brodersen, P., Petersen, M., Bjorn N.H., Zhu, S., Newman, M.A., Shokat, K.M., Rietz, S., Parker, J., and Mundy, J. (2006). Arabidopsis MAP kinase 4 regulates salicylic acid- and jasmonic acid/ethylene-dependent responses via EDS1 and PAD4. Plant J **47,** 532-546.

Chao, Q., Rothenberg, M., Solano, R., Roman, G., Terzaghi, W., and Ecker, J.R. (1997). Activation of the ethylene gas response pathway in Arabidopsis by the nuclear protein ETHYLENE-INSENSITIVE3 and related proteins. Cell **89,** 1133-1144.

Chen, Z., Gibson, T.B., Robinson, F., Silvestro, L., Pearson, G., Xu, B., Wright, A., Vanderbilt, C., and Cobb, M.H. (2001). MAP kinases. Chem Rev **101,** 2449-2476.

Cheong, Y.H., Moon, B.C., Kim, J.K., Kim, C.Y., Kim, M.C., Kim, I.H., Park, C.Y., Kim, J.C., Park, B.O., Koo, S.C., Yoon, H.W., Chung, W.S., Lim, C.O., Lee, S.Y., and Cho, M.J. (2003). BWMK1, a rice mitogen-activated protein kinase, locates in the nucleus and mediates pathogenesis-related gene expression by activation of a transcription factor. Plant Physiol **132,**

1961-1972.

Cowley, S., Paterson, H., Kemp, P., and Marshall, C.J. (1994). Activation of MAP kinase kinase is necessary and sufficient for PC12 differentiation and for transformation of NIH 3T3 cells. Cell **77,** 841-852.

Dai, Y., Wang, H., Li, B., Huang, J., Liu, X., Zhou, Y., Mou, Z., and Li, J. (2006). Increased expression of MAP KINASE KINASE7 causes deficiency in polar auxin transport and leads to plant architectural abnormality in Arabidopsis. Plant Cell **18,** 308-320.

Devoto, A., and Turner, J.G. (2003). Regulation of jasmonate-mediated plant responses in arabidopsis. Ann Bot (Lond) **92,** 329-337.

Devoto, A., Nieto-Rostro, M., Xie, D., Ellis, C., Harmston, R., Patrick, E., Davis, J., Sherratt, L., Coleman, M., and Turner, J.G. (2002). COI1 links jasmonate signalling and fertility to the SCF ubiquitin-ligase complex in Arabidopsis. Plant J **32,** 457-466.

Farmer, E.E., Almeras, E., and Krishnamurthy, V. (2003). Jasmonates and related oxylipins in plant responses to pathogenesis and herbivory. Curr Opin Plant Biol **6,** 372-378.

Feys, B., Benedetti, C.E., Penfold, C.N., and Turner, J.G. (1994). Arabidopsis mutants selected for resistance to the phytotoxin coronatine are male sterile, insensitive to methyl jasmonate, and resistant to a bacterial pathogen. Plant Cell **6,** 751-759.

Gomi, K., Ogawa, D., Katou, S., Kamada, H., Nakajima, N., Saji, H., Soyano, T., Sasabe, M., Machida, Y., Mitsuhara, I., Ohashi, Y., and Seo, S. (2005). A mitogen-activated protein kinase NtMPK4 activated by SIPKK is required for

jasmonic acid signaling and involved in ozone tolerance via stomatal movement in tobacco. Plant Cell Physiol **46,** 1902-1914.

He, P., Shan, L., Lin, N.C., Martin, G.B., Kemmerling, B., Nurnberger, T., and Sheen, J. (2006). Specific bacterial suppressors of MAMP signaling upstream of MAPKKK in Arabidopsis innate immunity. Cell **125,** 563-575.

Hoffman, N.E., Yang, S.F., and McKeon, T. (1982). Identification of 1-(malonylamino) cyclopropane-1-carboxylic acid as a major conjugate of 1-aminocyclopropane-1-carboxylic acid, an ethylene precursor in higher plants. Biochem Biophys Res Commun **104,** 765-770.

Huang, Y., Li, H., Hutchison, C.E., Laskey, J., and Kieber, J.J. (2003). Biochemical and functional analysis of CTR1, a protein kinase that negatively regulates ethylene signaling in Arabidopsis. Plant J **33,** 221-233.

Huang, Y., Li, H., Gupta, R., Morris, P.C., Luan, S., and Kieber, J.J. (2000). ATMPK4, an Arabidopsis homolog of mitogen-activated protein kinase, is activated in vitro by AtMEK1 through threonine phosphorylation. Plant Physiol **122,** 1301-1310.

Ichimura, K., Mizoguchi, T., Irie, K., Morris, P., Giraudat, J., Matsumoto, K., and Shinozaki, K. (1998). Isolation of ATMEKK1 (a MAP kinase kinase kinase)-interacting proteins and analysis of a MAP kinase cascade in Arabidopsis. Biochem Biophys Res Commun **253,** 532-543.

Ichimura, K., Shinozaki, K., Tena, G., Sheen, J., Henry, Y., Champion, A., Kreis, M., Zhang, S., Hirt, H., Wilson, C., Heberle-Bors, E., Ellis, B.E., Morris, P.C., Innes, R.W., Ecker, J.R., Scheel, D., Klessig, D.F., Machida, Y., Mundy, J., Ohashi, Y., and Walker, J.C. (2002). Mitogen-activated protein

kinase cascades in plants: a new nomenclature. Trends Plant Sci **7,** 301-308.

Kieber, J.J., Rothenberg, M., Roman, G., Feldmann, K.A., and Ecker, J.R. (1993). CTR1, a negative regulator of the ethylene response pathway in Arabidopsis, encodes a member of the raf family of protein kinases. Cell **72,** 427-441.

Kim, C.Y., Liu, Y., Thorne, E.T., Yang, H., Fukushige, H., Gassmann, W., Hildebrand, D., Sharp, R.E., and Zhang, S. (2003). Activation of a stress-responsive mitogen-activated protein kinase cascade induces the biosynthesis of ethylene in plants. Plant Cell **15,** 2707-2718.

Kumar, D., and Klessig, D.F. (2000). Differential induction of tobacco MAP kinases by the defense signals nitric oxide, salicylic acid, ethylene, and jasmonic acid. Mol Plant Microbe Interact **13,** 347-351.

Leon, J., Rojo, E., and Sanchez-Serrano, J.J. (2001). Wound signalling in plants. J Exp Bot **52,** 1-9.

Liu, Y., and Zhang, S. (2004). Phosphorylation of 1-aminocyclopropane-1-carboxylic acid synthase by MPK6, a stress-responsive mitogen-activated protein kinase, induces ethylene biosynthesis in Arabidopsis. Plant Cell **16,** 3386-3399.

Lorenzo, O., Piqueras, R., Sanchez-Serrano, J.J., and Solano, R. (2003). ETHYLENE RESPONSE FACTOR1 integrates signals from ethylene and jasmonate pathways in plant defense. Plant Cell **15,** 165-178.

Lorenzo, O., Chico, J.M., Sanchez-Serrano, J.J., and Solano, R. (2004). JASMONATE-INSENSITIVE1 encodes a MYC transcription factor essential to discriminate between different jasmonate-regulated defense responses in Arabidopsis. Plant Cell **16,** 1938-1950.

Lu, C., Han, M.H., Guevara-Garcia, A., and Fedoroff, N.V. (2002).

Mitogen-activated protein kinase signaling in postgermination arrest of development by abscisic acid. Proc Natl Acad Sci USA **99,** 15812-15817.

Madhani, H.D., and Fink, G.R. (1998). The riddle of MAP kinase signaling specificity. Trends Genet **14,** 151-155.

Mansour, S.J., Matten, W.T., Hermann, A.S., Candia, J.M., Rong, S., Fukasawa, K., Vande W.G.F., and Ahn, N.G. (1994). Transformation of mammalian cells by constitutively active MAP kinase kinase. Science **265,** 966-970.

Marshall, C.J. (1994). MAP kinase kinase kinase, MAP kinase kinase and MAP kinase. Curr Opin Genet Dev **4,** 82-89.

Matsuoka, D., Nanmori, T., Sato, K., Fukami, Y., Kikkawa, U., and Yasuda, T. (2002). Activation of AtMEK1, an Arabidopsis mitogen-activated protein kinase kinase, in vitro and in vivo: analysis of active mutants expressed in *E. coli* and generation of the active form in stress response in seedlings. Plant J **29,** 637-647.

McConn, M., Creelman, R.A., Bell, E., Mullet, J.E., and Browse, J. (1997). Jasmonate is essential for insect defense in Arabidopsis. Proc Natl Acad Sci USA **94,** 5473-5477.

Menke, F.L., van Pelt, J.A., Pieterse, C.M., and Klessig, D.F. (2004). Silencing of the mitogen-activated protein kinase MPK6 compromises disease resistance in Arabidopsis. Plant Cell **16,** 897-907.

Miles, G.P., Samuel, M.A., Zhang, Y., and Ellis, B.E. (2005). RNA interference-based (RNAi) suppression of AtMPK6, an Arabidopsis mitogen-activated protein kinase, results in hypersensitivity to ozone and misregulation of AtMPK3. Environ Pollut **138,** 230-237.

Mizoguchi, T., Hayashida, N., Yamaguchi-Shinozaki, K., Kamada, H., and Shinozaki, K. (1993). ATMPKs: a gene family of plant MAP kinases in *Arabidopsis thaliana*. FEBS Lett **336,** 440-444.

Mizoguchi, T., Ichimura, K., Irie, K., Morris, P., Giraudat, J., Matsumoto, K., and Shinozaki, K. (1998). Identification of a possible MAP kinase cascade in *Arabidopsis thaliana* based on pairwise yeast two-hybrid analysis and functional complementation tests of yeast mutants. FEBS Lett **437,** 56-60.

Nakagami, H., Pitzschke, A., and Hirt, H. (2005). Emerging MAP kinase pathways in plant stress signalling. Trends Plant Sci **10,** 339-346.

Penninckx, I.A., Thomma, B.P., Buchala, A., Metraux, J.P., and Broekaert, W.F. (1998). Concomitant activation of jasmonate and ethylene response pathways is required for induction of a plant defensin gene in Arabidopsis. Plant Cell **10,** 2103-2113.

Petersen, M., Brodersen, P., Naested, H., Andreasson, E., Lindhart, U., Johansen, B., Nielsen, H.B., Lacy, M., Austin, M.J., Parker, J.E., Sharma, S.B., Klessig, D.F., Martienssen, R., Mattsson, O., Jensen, A.B., and Mundy, J. (2000). Arabidopsis map kinase 4 negatively regulates systemic acquired resistance. Cell **103,** 1111-1120.

Posas, F., and Saito, H. (1997). Osmotic activation of the HOG MAPK pathway via Ste11p MAPKKK: scaffold role of Pbs2p MAPKK. Science **276,** 1702-1705.

Ren, D., Yang, H., and Zhang, S. (2002). Cell death mediated by MAPK is associated with hydrogen peroxide production in Arabidopsis. J Biol Chem **277,** 559-565.

Rojo, E., Leon, J., and Sanchez-Serrano, J.J. (1999). Cross-talk between wound signalling pathways determines local versus systemic gene expression in

Arabidopsis thaliana. Plant J **20,** 135-142.

Rojo, E., Titarenko, E., Leon, J., Berger, S., Vancanneyt, G., and Sanchez-Serrano, J.J. (1998). Reversible protein phosphorylation regulates jasmonic acid-dependent and -independent wound signal transduction pathways in *Arabidopsis thaliana*. Plant J **13,** 153-165.

Romeis, T., Piedras, P., Zhang, S., Klessig, D.F., Hirt, H., and Jones, J.D. (1999). Rapid Avr9- and Cf-9 -dependent activation of MAP kinases in tobacco cell cultures and leaves: convergence of resistance gene, elicitor, wound, and salicylate responses. Plant Cell **11,** 273-287.

Sasaki, Y., Asamizu, E., Shibata, D., Nakamura, Y., Kaneko, T., Awai, K., Amagai, M., Kuwata, C., Tsugane, T., Masuda, T., Shimada, H., Takamiya, K., Ohta, H., and Tabata, S. (2001). Monitoring of methyl jasmonate-responsive genes in Arabidopsis by cDNA macroarray: self-activation of jasmonic acid biosynthesis and crosstalk with other phytohormone signaling pathways. DNA Res **8,** 153-161.

Seo, S., Sano, H., and Ohashi, Y. (1999). Jasmonate-based wound signal transduction requires activation of WIPK, a tobacco mitogen-activated protein kinase. Plant Cell **11,** 289-298.

Seo, S., Okamoto, M., Seto, H., Ishizuka, K., Sano, H., and Ohashi, Y. (1995). Tobacco MAP kinase: a possible mediator in wound signal transduction pathways. Science **270,** 1988-1992.

Shibata, W., Banno, H., Ito, Y., Hirano, K., Irie, K., Usami, S., Machida, C., and Machida, Y. (1995). A tobacco protein kinase, NPK2, has a domain homologous to a domain found in activators of mitogen-activated protein

kinases (MAPKKs). Mol Gen Genet **246,** 401-410.

Staswick, P.E., Su, W., and Howell, S.H. (1992). Methyl jasmonate inhibition of root growth and induction of a leaf protein are decreased in an *Arabidopsis thaliana* mutant. Proc Natl Acad Sci USA **89,** 6837-6840.

Stepanova, A.N., and Alonso, J.M. (2005). Ethylene signaling pathway. Sci STKE **2005,** cm3.

Teige, M., Scheikl, E., Eulgem, T., Doczi, R., Ichimura, K., Shinozaki, K., Dangl, J.L., and Hirt, H. (2004). The MKK2 pathway mediates cold and salt stress signaling in Arabidopsis. Mol Cell **15,** 141-152.

The Arabidopsis Genome Initiative. (2000). Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. Nature **408**, 796-815.

Thomma, B.P., Nelissen, I., Eggermont, K., and Broekaert, W.F. (1999). Deficiency in phytoalexin production causes enhanced susceptibility of *Arabidopsis thaliana* to the fungus *Alternaria brassicicola*. Plant J **19,** 163-171.

Tuominen, H., Overmyer, K., Keinanen, M., Kollist, H., and Kangasjarvi, J. (2004). Mutual antagonism of ethylene and jasmonic acid regulates ozone-induced spreading cell death in Arabidopsis. Plant J **39,** 59-69.

Turner, J.G., Ellis, C., and Devoto, A. (2002). The jasmonate signal pathway. Plant Cell **14 Suppl,** S153-164.

Wang, K.L., Li, H., and Ecker, J.R. (2002). Ethylene biosynthesis and signaling networks. Plant Cell **14 Suppl,** S131-151.

Widmann, C., Gibson, S., Jarpe, M.B., and Johnson, G.L. (1999). Mitogen-activated protein kinase: conservation of a three-kinase module from yeast to human. Physiol Rev **79,** 143-180.

Xie, D.X., Feys, B.F., James, S., Nieto-Rostro, M., and Turner, J.G. (1998). COI1: an Arabidopsis gene required for jasmonate-regulated defense and fertility. Science **280,** 1091-1094.

Xu, L., Liu, F., Lechner, E., Genschik, P., Crosby, W.L., Ma, H., Peng, W., Huang, D., and Xie, D. (2002). The SCF(COI1) ubiquitin-ligase complexes are required for jasmonate response in Arabidopsis. Plant Cell **14,** 1919-1935.

Xu, Y., Chang, P., Liu, D., Narasimhan, M.L., Raghothama, K.G., Hasegawa, P.M., and Bressan, R.A. (1994). Plant defense genes are synergistically induced by ethylene and methyl jasmonate. Plant Cell **6,** 1077-1085.

Yamaguchi-Shinozaki, K., and Shinozaki, K. (1994). A novel cis-acting element in an Arabidopsis gene is involved in responsiveness to drought, low-temperature, or high-salt stress. Plant Cell **6,** 251-264.

Yang, K.Y., Liu, Y., and Zhang, S. (2001). Activation of a mitogen-activated protein kinase pathway is involved in disease resistance in tobacco. Proc Natl Acad Sci USA **98,** 741-746.

Zhang, S., and Klessig, D.F. (1997). Salicylic acid activates a 48-kD MAP kinase in tobacco. Plant Cell **9,** 809-824.

Zhang, S., Du, H., and Klessig, D.F. (1998). Activation of the tobacco SIP kinase by both a cell wall-derived carbohydrate elicitor and purified proteinaceous elicitins from *Phytophthora* spp. Plant Cell **10,** 435-450.

Tables and Figures

Table 1. Prominent upregulated genes in steroid-inducible *MKK3DD* plants.

			Experiment 1[a] (Transgenic line 5)		Experiment 2 (Transgenic line 3)	
	AGI number[b]	Discription[c]	Fold change[d]	P-value[e]	Fold change[d]	P-value[e]
Metabolism	At1g43800	stearoyl acyl carrier protein desaturase, putative	5.6	1.6E-24		
	At3g45140	lipoxygenase AtLOX2			7.0	4.11E-27
	At4g12290	copper amine oxidase -like protein	2.0	3.4E-08	5.3	1.34E-22
	At1g05530	UDP-glycosyltransferase family			7.3	2.60E-26
	At1g05560	UDP-glucose transferase(UGT1)	1.7	1.3E-07	8.1	2.02E-28
	At1g55850	cellulose synthase catalytic subunit, putative			5.4	2.52E-23
	At2g29420	glutathione transferase, putative			6.2	4.07E-26
	At5g07560	glycine-rich protein GRP20	5.6	7.9E-25	2.3	8.45E-09
	At5g20740	ripening-related protein - like	24.2	2.4E-33	5.2	2.95E-24
	At5g50030	putative protein	5.3	1.1E-23		
Transcription	At5g66320	GATA zinc finger protein	6.5	1.2E-24	1.5	1.72E-03
	At1g01300	chloroplast nucleoid DNA binding protein, putative	5.9	5.9E-23	1.7	5.24E-03
	At1g01720	NAC domain protein, putative (ATAF1)	5.1	3.7E-21	3.0	6.86E-14
	At1g53170	ethylene responsive element binding factor 8	5.9	2.7E-25	1.4	1.26E-03
Protein fate	At4g33490	nucellin -like protein			5.6	8.53E-24
Transport	At2g38170	high affinity Ca2+ antiporter			5.4	5.80E-24
	At2g38540	putative nonspecific lipid-transfer protein	8.1	1.5E-27	1.9	3.13E-06
	At4g13510	ammonium transport protein (AMT1)	6.7	5.8E-26	2.7	8.51E-13
	At5g59310	nonspecific lipid-transfer protein precursor - like	12.2	1.6E-31		
Signal transduction	At1g10430	serine/threonine protein phosphatase, PP2A	6.2	1.3E-05		
	At2g36570	leucine-rich repeat transmembrane protein kinase, putative	6.0	1.4E-25	1.6	3.00E-05
Defense	At1g52400	glycosyl hydrolase family 1, beta-glucosidase (BG1)			6.3	1.45E-24
	At1g75940	glycosyl hydrolase family 1, beta-glucosidase(ATA27)	3.3	4.4E-08	21.3	8.04E-32
	At2g21130	cyclophilin (CYP2)	5.8	1.8E-23	2.9	6.43E-12
	At2g28190	copper/zinc superoxide dismutase (CSD2)	5.1	1.6E-24	1.1	1.27E-01
	At5g25610	dehydration-induced protein RD22	5.7	2.9E-24	1.2	2.21E-01
	At5g44420	plant defensin protein, putative (PDF1.2a)	12.6	3.9E-31	6.1	2.69E-25
Unknown	At1g12570	hypothetical protein	6.3	7.3E-26	2.6	4.32E-09
	At1g19960	hypothetical protein	6.5	6.4E-27	1.3	9.88E-05
	At1g78020	expressed protein	5.5	3.9E-25	2.2	6.46E-12
	At2g20870	expressed protein	5.9	2.9E-25	2	2.87E-08
	At2g25510	expressed protein	5.1	8.2E-23	2.3	1.05E-09
	At3g04720	hevein-like protein precursor (PR-4)	5.7	7.3E-23		
	At3g15400	anther development protein, ATA20	9.8	1.2E-28	6.1	1.26E-23
	At3g22240	expressed protein	6.3	4.2E-26	1.5	4.91E-04
	At3G57690	arabinogalactan-protein, putative (AGP23)	5.7	3.9E-25		
	At5g20790	putative protein	6.1	1.1E-25		
	At5g22430	expressed protein	6.3	1.6E-26	5.4	4.14E-22
	At5g22460	putative protein	1.6	9.5E-05	5.4	9.17E-21

[a] Upregulated genes in DEX-inducible *MKK3DD* transgenic plants (*MKK3DD*/Cont.).

[b] AGI, Arabidopsis Genome Initiative.

[c] Description as given by The Institute for Genomic Research database.

[d] Median of fold change

[e] P values < 0.001 were studied.

Table 2. Prominent upregulated genes in steroid-inducible *MKK4DD* plants.

			Experiment 1[a] (Transgenic line 7)		Experiment 2 (Transgenic line 2)	
	AGI number[b]	Discription[c]	Fold change[d]	P-value[e]	Fold change[d]	P-value[e]
Metabolism	At1g02920	glutathione transferase, putative	7.9	1.8E-28	6.1	8.7E-26
	At1g02930	glutathione transferase, putative (GST1)	23.0	3.6E-33	8.2	2.5E-28
	At3g54640	tryptophan synthase alpha chain	6.1	4.1E-26	3.2	1.7E-18
	At4g11280	ACC synthase (AtACS-6)	8.4	6.3E-28	2.3	1.8E-08
	At4g15210	glycosyl hydrolase family 14 (beta-amylase)	5.3	7.5E-20	1.1	2.9E-01
	At4g20830	FAD-linked oxidoreductase family	5.1	7.5E-22	2.1	6.5E-07
	At4g27070	tryptophan synthase beta-subunit (TSB2)	7.6	3.0E-28	2.4	5.2E-14
	At4g39940	adenosine-5-phosphosulfate-kinase	6.6	1.6E-26	4.6	1.0E-22
	At5g38900	frnE protein - like	10.1	6.9E-29	3.5	3.9E-18
	At5g38910	germin - like protein	6.7	9.7E-26		
	At5g57220	cytochrome p450 family	9.5	2.3E-24	9.6	1.3E-26
	At5g65140	trehalose-6-phosphate phosphatase	6.5	7.2E-24	2.8	3.1E-09
	At1g21100	O-methyltransferase 1, putative	7.3	2.4E-27	5.2	9.2E-24
	At1g21110	O-methyltransferase 1, putative	5.5	1.7E-22	2.0	7.3E-07
	At1g21120	O-methyltransferase 1, putative	8.5	1.5E-27	3.8	2.3E-17
	At1g21130	O-methyltransferase 1, putative	5.3	3.7E-24	2.5	1.2E-12
	At1g23730	putative carbonic anhydrase	6.4	2.3E-25	1.3	7.8E-03
	At1g26380	FAD-linked oxidoreductase family	23.6	3.1E-33	13.5	5.6E-31
	At1g26410	FAD-linked oxidoreductase family	5.3	1.1E-23	5.4	2.3E-21
	At1g26420	FAD-linked oxidoreductase family	17.0	2.2E-31	11.2	6.1E-28
	At1g69920	glutathione transferase, putative	5.7	7.4E-25	3.2	1.2E-14
	At1g74590	glutathione transferase, putative	6.1	4.3E-25	2.7	3.4E-14
	At2g02010	glutamate decarboxylase	29.4	2.8E-33	21.1	2.5E-30
	At2g04400	putative indole-3-glycerol phosphate synthase	6.7	2.0E-25	3.4	3.0E-16
	At2g29350	putative tropinone reductase	15.3	2.6E-31	1.7	4.3E-04
	At2g29720	monooxygenase family	7.7	7.9E-27	3.3	1.2E-16
	At5g40990	gDSL-motif lipase/hydrolase-like protein	3.6	3.5E-15	6.0	6.7E-24
	At2g26560	similar to latex allergen from Hevea brasiliensis	6.8	9.1E-27	3.9	1.7E-20
Transcription	At2g38470	WRKY family transcription factor	5.1	1.9E-23	2.8	1.2E-14
	At5g13080	WRKY family transcription factor	6.5	1.2E-26	2.6	3.8E-14
	At1g18570	myb family transcription factor	18.8	2.0E-32	7.6	6.0E-27
	At1g27730	salt-tolerance zinc finger protein	5.7	4.2E-25	2.6	2.5E-14
	At1g74080	putative transcription factor	5.5	1.7E-21	3.5	2.1E-11
	At1g80840	WRKY family transcription factor	7.4	5.4E-27	3.8	1.1E-19
	At2g43000	NAM (no apical meristem)-like protein	8.4	2.2E-22	6.8	9.2E-25
	At4g23810	WRKY family transcription factor	6.2	1.5E-26	3.4	2.0E-17
Protein fate	At3g12580	heat shock protein 70	5.8	2.1E-24	1.7	3.0E-06
	At1g24140	putative metalloproteinase	8.1	5.5E-27	4.6	1.2E-23
	At2g31980	putative cysteine proteinase inhibitor B (cystatin B)	6.7	1.3E-25	1.7	3.0E-05
	At2g32020	putative alanine acetyl transferase	5.0	2.3E-23	1.9	7.3E-08
	At2g38860	expressed protein	6.0	4.9E-25	3.9	2.3E-18
Transport	At4g13420	potassium transporter - like protein	12.5	5.4E-31	1.2	3.1E-01
	At1g74360	leucine-rich repeat transmembrane protein kinase, putative	6.2	1.4E-24	3.9	4.1E-18
	At3g28510	hypothetical protein	5.9	5.6E-25		
	At4g12120	putative protein	5.2	3.7E-18	2.1	2.4E-06
	At4g39670	putative protein	7.2	5.3E-25	1.9	2.4E-06
Signal transduction	At2g19190	putative receptor-like protein kinase	8.9	6.0E-27	1.6	2.4E-03
	At3g16530	putative lectin	15.6	1.3E-31	5.5	1.7E-23
	At5g25930	receptor-like protein kinase - like	9.9	9.3E-30	5.1	1.2E-24
	At5g18470	putative protein	5.6	2.8E-22	4.2	2.6E-17
Defense	At2g43590	glycosyl hydrolase family 19 (chitinase)	9.7	3.5E-29	2.9	9.4E-16
	At3g47540	glycosyl hydrolase family 19 (chitinase)	12.7	3.3E-30	3.1	7.8E-14
	At4g14630	germin precursor oxalate oxidase	5.7	5.3E-24		
	At5g02780	In2-1 protein, putative	5.2	6.5E-21	2.7	2.5E-09
	At5g47910	respiratory burst oxidase protein	5.4	1.6E-23	1.9	3.2E-06
	At1g14540	anionic peroxidase, putative	6.2	1.3E-24	2.6	1.5E-03
	At1g57630	disease resistance protein (TIR class), putative	18.8	8.9E-32	2.9	1.0E-12
	At1g61810	glycosyl hydrolase family 1	11.7	1.3E-27	4.2	1.2E-15
	At1g65970	type 2 peroxiredoxin, putative	22.5	4.3E-33	6.7	6.1E-27

(continued to next page)

	AGI number[b]	Discription[c]	Experiment 1[a] (Transgenic line 7) Fold change[d]	P-value[e]	Experiment 2 (Transgenic line 2) Fold change[d]	P-value[e]
	At2g15220	expressed protein	5.0	6.2E-23	3.6	1.3E-19
	At2g43570	glycosyl hydrolase family 19 (chitinase)	12.9	3.9E-31	4.7	3.4E-21
Unknown	At1g13520	hypothetical protein	6.7	2.1E-25	3.1	1.8E-13
	At1g24150	unknown protein	6.2	5.6E-23	2.3	4.0E-11
	At1g25083	F5A9.7	7.5	1.6E-27	3.7	1.5E-19
	At1g28190	hypothetical protein	9.6	4.2E-29	4.0	4.3E-20
	At1g55450	expressed protein	5.6	2.7E-24	3.1	1.1E-16
	At1g78410	expressed protein	7.9	1.4E-26	6.1	5.7E-22
	At2g18660	hypothetical protein	5.7	1.9E-18		
	At2g18690	expressed protein	18.1	9.6E-32	4.3	4.5E-19
	At2g30750	cytochrome p450 family	28.7	1.4E-32	19.3	8.4E-32
	At3g01830	expressed protein	14.5	8.6E-30	3.2	1.4E-08
	At3g02840	expressed protein	7.4	2.3E-26	6.5	5.6E-22
	At3g10500	expressed protein	5.1	3.3E-23	2.2	1.7E-09
	At3g15356	similar to putative lectin	12.6	3.9E-30	5.5	5.0E-23
	At3g26830	cytochrome p450 family	49.4	1.3E-34	19.0	1.8E-32
	At3g50480	expressed protein	5.2	2.5E-23	2.0	5.5E-08
	At3g54150	embryonic abundant protein -like	9.3	2.5E-28	3.7	4.7E-18
	At4g33050	putative protein	7.1	7.9E-26	3.6	2.2E-19
	At4g37290	expressed protein	5.4	5.2E-18	7.3	5.1E-25
	At4g37370	cytochrome p450 family	11.5	3.1E-30	5.9	9.8E-25
	At4g39950	cytochrome P450 - like protein	11.6	5.4E-30	5.6	6.3E-23
	At5g52760	expressed protein	5.9	8.7E-21	1.6	7.6E-01
	At1g67990	caffeoyl-CoA 3-O-methyltransferase, putative	11.3	2.1E-28	9.4	1.2E-18

[a] Upregulated genes in DEX-inducible *MKK4DD* transgenic plants (*MKK4DD*/Cont.).

[b] AGI, Arabidopsis Genome Initiative.

[c] Description as given by The Institute for Genomic Research database.

[d] Median of fold change

[e] P values < 0.001 were studied.

Table 3. Prominent upregulated genes both in steroid-inducible *MKK3DD* and *MKK4DD* plants.

AGI number[c]	Discription[d]	Experiment 1[a] (Transgenic line 5)		Experiment 2 (Transgenic line 3)		Experiment 1[b] (Transgenic line 7)		Experiment 2 (Transgenic line 2)	
		Fold change[e]	P-value[f]	Fold change[e]	P-value[f]	Fold change[e]	P-value[f]	Fold change[e]	P-value[f]
At4g23600	tyrosine transaminase like protein	3.2	9.5E-19	8.7	3.43E-28	2.8	2.1E-15	5.1	1.7E-23
At5g07550	glycine-rich protein GRP19	11.3	7.5E-28	14.1	2.75E-23	5.7	6.7E-18	9.5	2.5E-10
At1g05680	putative indole-3-acetate beta-glucosyltransferase			12.7	3.49E-31	8.8	5.9E-29	6.4	1.3E-26
At5g07530	glycine-rich protein GRP17	9.1	1.5E-25	21.6	1.92E-32	21.5	1.2E-32	14.9	9.0E-31
At5g24780	vegetative storage protein Vsp1	14.4	1.6E-31	6.8	1.19E-26	6.9	1.3E-26	3.1	3.9E-17
At5g27420	RING-H2 zinc finger protein-like	6.6	3.3E-25	1.3	6.05E-02	12.1	9.5E-30	4.5	2.6E-18
At5g37260	putative protein	2.2	1.0E-10	10.2	2.60E-29			9.5	1.4E-29
At5g61160	anthocyanin 5-aromatic acyltransferase - like protein	3.7	4.1E-19	7.6	2.10E-26	11.7	1.4E-29	8.9	1.0E-28
At1g66850	lipid transfer protein, putative	7.4	8.6E-28	9.0	1.64E-27	5.6	1.7E-24	4.3	3.2E-20
At5g60100	pseudo-response regulator - like	1.6	1.3E-03	5.4	1.85E-23	2	1.0E-06	7.3	1.5E-26
At4g30650	low temperature and salt responsive protein homolog	5.3	3.0E-23	5.0	1.80E-22	2.2	8.0E-09	5.2	1.6E-23
At5g15970	cold-regulated protein COR6.6 (KIN2)	12.2	1.0E-30	7.4	3.42E-27	3.8	8.0E-20	6.9	1.1E-26
At5g23240	putative protein			6.7	4.62E-26	1.6	8.0E-05	5.6	2.5E-23
At1g07050	expressed protein	3.1	3.5E-12	12.7	3.79E-30			11.3	3.3E-29
At2g42530	cold-regulated protein cor15b precursor	2.3	1.4E-06	7.5	3.98E-27	2.7	3.6E-09	8.7	2.1E-27
At4g34950	putative protein	5.9	7.1E-25	6.3	9.16E-25	1.7	9.8E-06	5.1	5.7E-24
At5g42900	putative protein	1.4	1.5E-04	6.9	2.69E-27			5.8	5.7E-26

[a] Upregulated genes in DEX-inducible *MKK3DD* transgenic plants (*MKK3DD*/Cont.).

[b] Upregulated genes in DEX-inducible *MKK4DD* transgenic plants (*MKK4DD*/Cont.).

[c] AGI, Arabidopsis Genome Initiative.

[d] Description as given by The Institute for Genomic Research database.

[e] Median of fold change

[f] P values < 0.001 were studied.

Table 4. steroid-inducible overexpressors of *MKK3DD* does not lead to an increase in JA levels.

Expt.	Time after +DEX (h)	JA (ng/g F.W.)	
		Cont.	*MKK3DD*
I	0	24.7	22.85
	24	27.36	14.07
II	0	19.07	9.73
	24	24.62	8.57

Transgenic *MKK3DD* and control plants were sprayed with 10 μM DEX. Whole plants were collected at the times indicated after DEX treatment, and JA levels were determined as described in Methods.

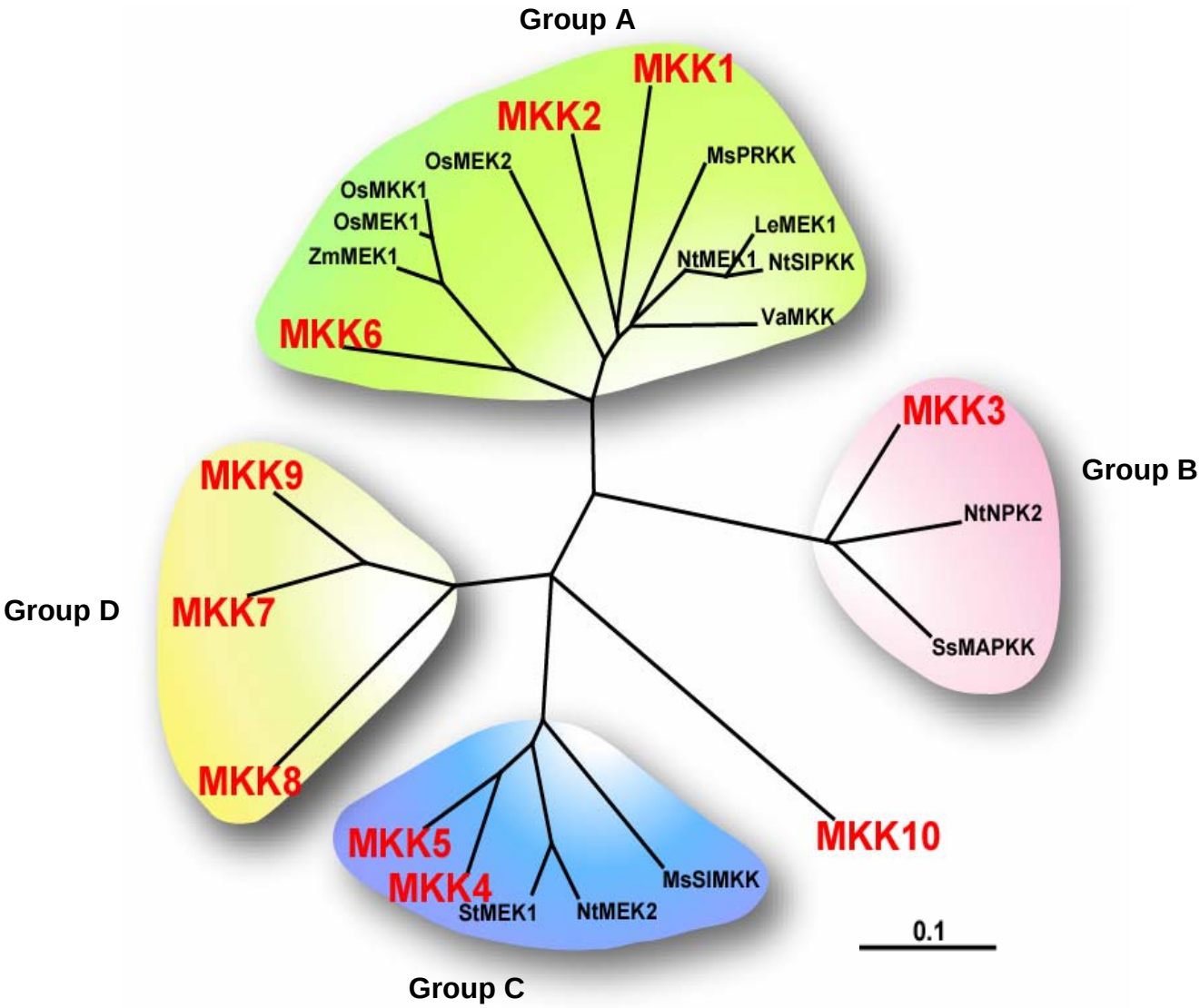

Figure 1. Phylogenetic tree of plant MAP kinase kinase.
The neighbor-joining phylogenetic tree was created using ClustalW on the DNA Data Bank of Japan and DendroMaker. Full length amino acid sequences of plant MAPKK from 9 species. *Arabidopsis* MAPKKs are shown in red bold letters. To identify the species of origin for each gene name, Le, *Lycopersicon esculentum*; Ms, *Medicago sativa*; Nt, *Nicotiana tabacum*; Os, *Oryza sativa*; Ss, *Suaeda salsa*; St, *Solanum tuberosum*; Va, *Vitis aestivalis*; Zm, *Zea mays*.

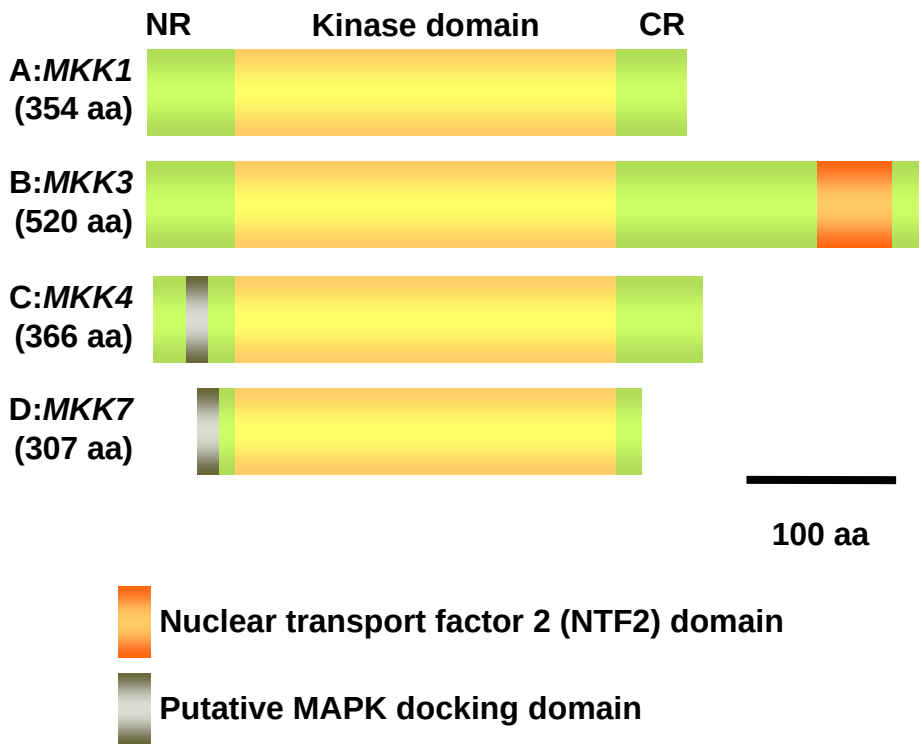

Figure 2. Domain structures of *MKK1*, *MKK3*, *MKK4*, and *MKK7*.
Scanning of the protein sequences for the presence of known motifs and domains was performed at the website PlantsP (http://plantsp.sdsc.edu/). Scale bar = 100 amino acids.

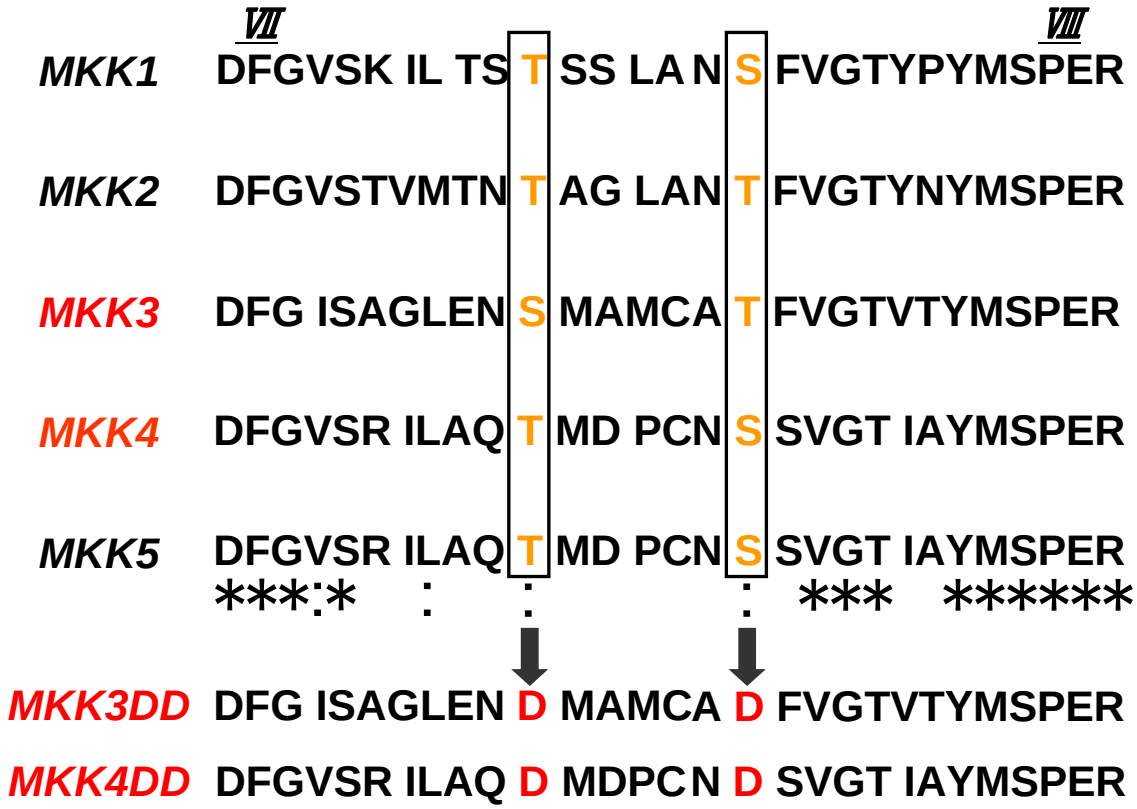

Figure 3. Comparison of the deduced amino acid sequences around kinase subdomains Ⅶ to Ⅷ between *Arabidopsis* MAPKKs.
Boxed amino acids correspond to the regulatory phosphorylation consensus sequence "S/TxxxxxS/T". These amino acids represent those of putative phosphorylation site of plant MAPKKs.

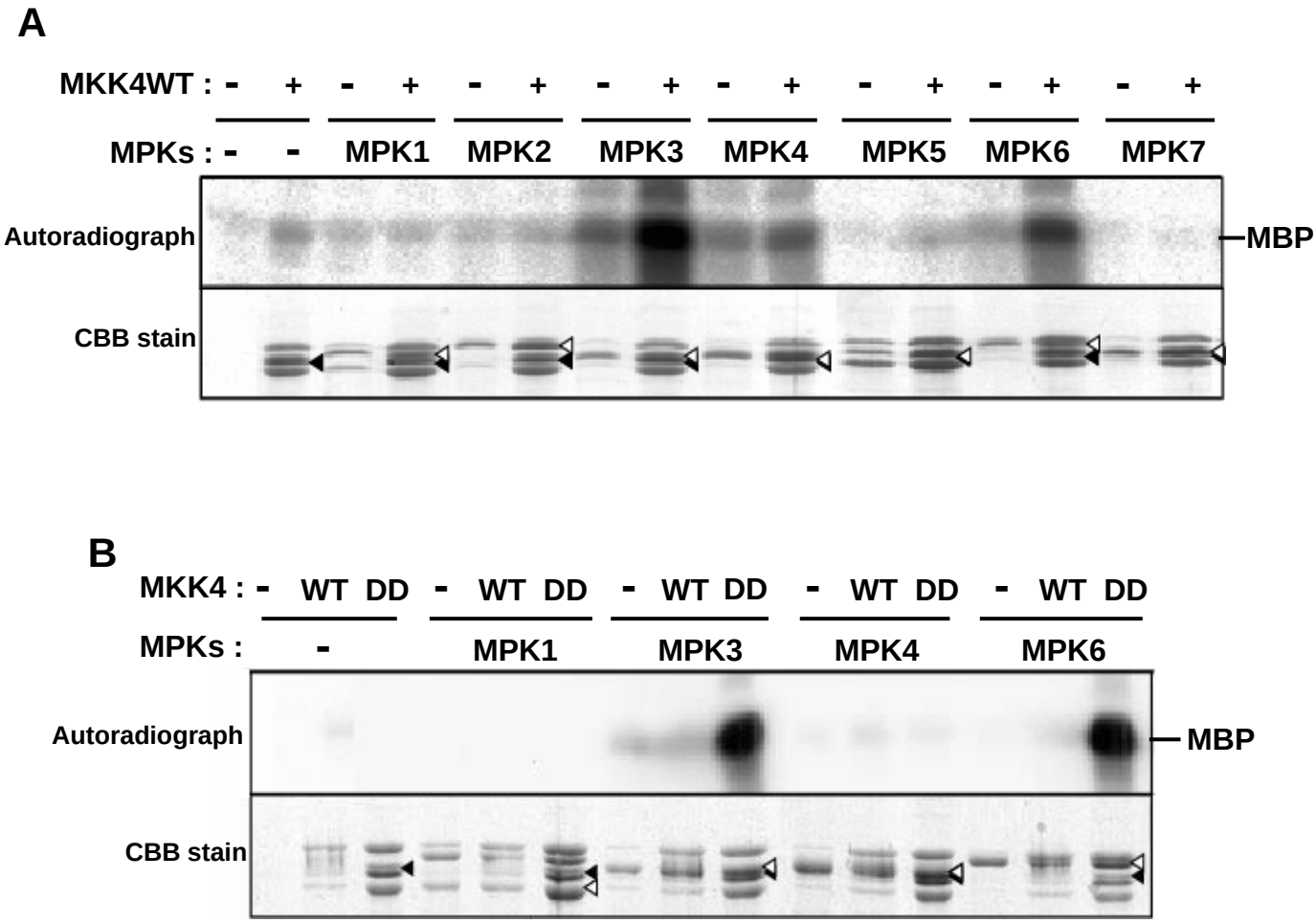

Figure 4. Specific activation of MAPKs by MKK4.
(A) Affinity purified GST-fusion MAPKs and MKK4 expressed in *E. coli* were used for *in vitro* activation assay. MKK4WT (1 μg) was incubated with (+) or without (-) each MPKs (1 μg) in the kinase reaction mixture containing myelin basic protein (MBP), and aliquots of the samples were separated on SDS-PAGE and subjected to autoradiography (Autoradiograph). A Coomassie stain of the MPKs (white heads arrow) and MKK4WT (black heads arrow) is shown in the lower panel (CBB stain). (B) Constitutive active MKK4DD activates MPK3 and MPK6. *in vitro* activation assay using either wild-type MKK4 (WT) or constitutive active MKK4DD (DD) tested activation of MPK6. Kinase activity of each MAPKs was measured with MBP.

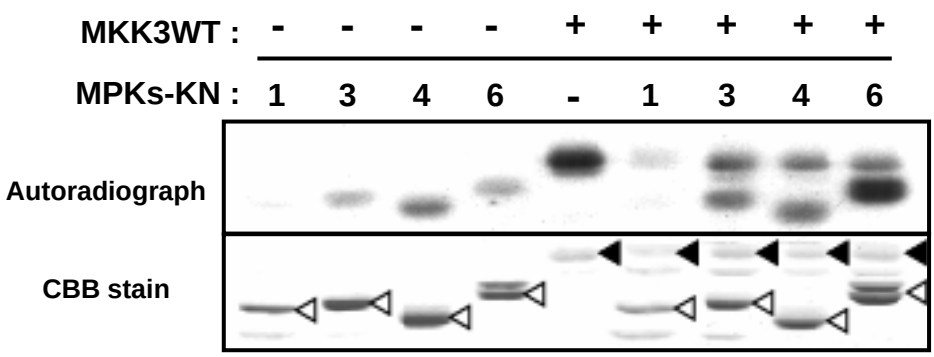

Figure 5. Autophosphorylation of recombinant MPKs and MKK3 proteins, and phosphorylation of MPKs by MKK3.

Affinity purified inactive GST-fusion MAPKs (MPKs-KN) and GST-MKK3 (MKK3WT) were expressed in *E. coli.* MKK3WT (1 μg) was incubated each inactive MPK (1 μg) in the kinase reaction mixture, and aliquots of the samples were separated on SDS-PAGE and subjected to autoradiography. A Coomassie stain of the MPKs-KN (white heads arrow) and MK3WT (black heads arrow) are shown in the lower panel (CBB stain).

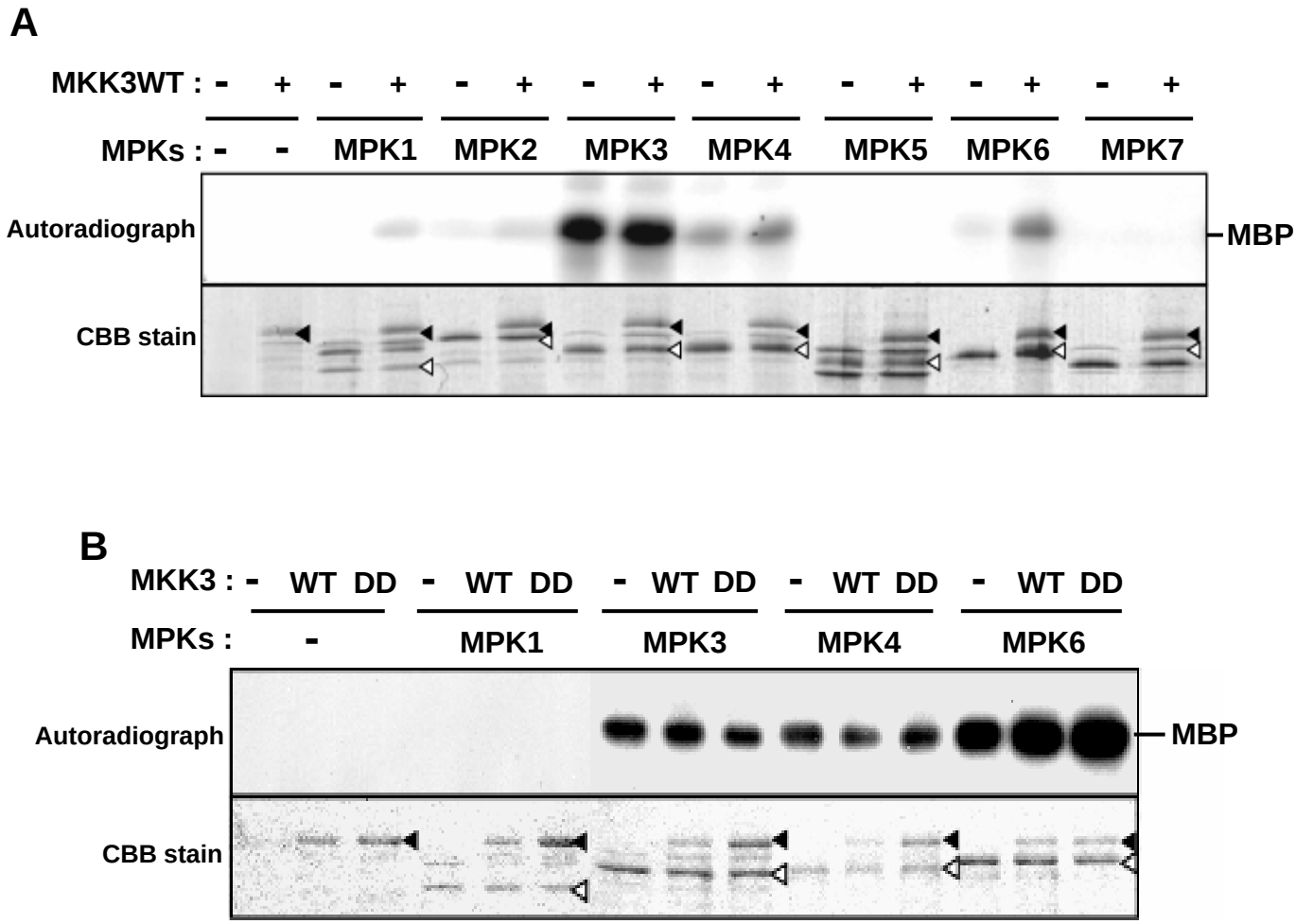

Figure 6. Specific activation of MAPKs by MKK3.
(A) Affinity purified GST-fusion MAPKs and MKK3 expressed in *E. coli* were used for *in vitro* activation assay. MKK3WT (1 μg) was incubated with (+) or without (-) each MPKs (1 mg) in the kinase reaction mixture containing myelin basic protein (MBP), and aliquots of the samples were separated on SDS-PAGE and subjected to autoradiography (Autoradiograph). A Coomassie stain of the MPKs (white arrow head) and MKK4WT (black arrow head) is shown in the lower panel (CBB stain).
(B) Constitutive active MKK3DD activates MPK6. *in vitro* activation assay using either wild-type MKK3 (WT) or constitutive active MKK3DD (DD) tested activation of MPK6. Kinase activity of each MAPKs was measured with MBP.

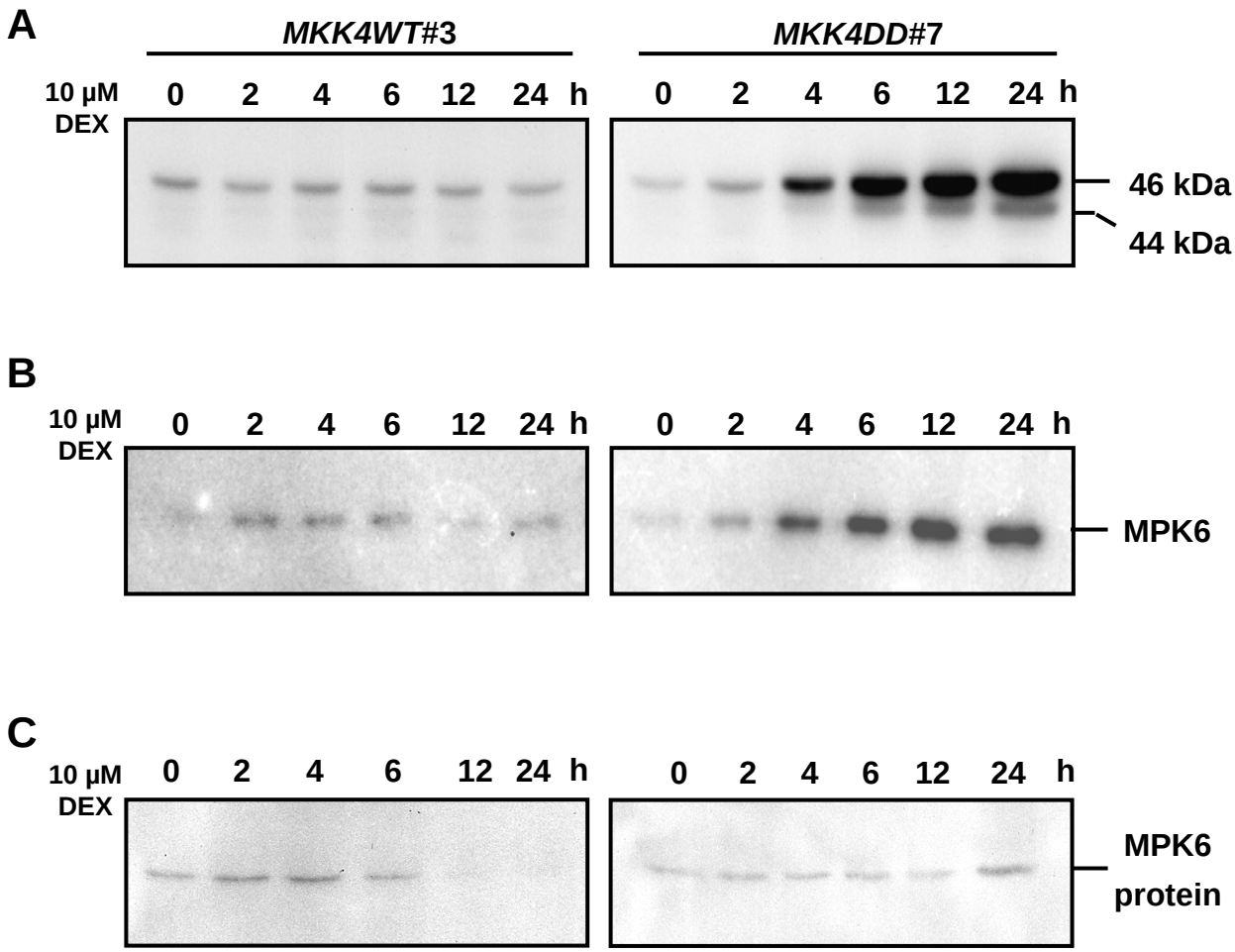

Figure 7. Enhanced expression of *MKK4DD* activates MPK6 *in vivo*.
Induction of *MKK4DD* expression by DEX activates endogenous MPK6. Dexamethazone inducible transgenic plants carrying the constitutive active MAPKK transgene *MKK4DD* was treated with 10 μM DEX. As controls, transgenic plant carrying the wild-type MAPKK transgene *MKK4WT* was treated side by side.
(A) Whole tissues were collected at indicated times and protein extracts were prepared. The activation of endogenous 46 kDa proteins was determined by in-gel kinase assay with MBP as the substrate.
(B) The activation of endogenous MPK6 was determined by in-gel kinase assay using MPK6-specific antibody, Ab6NT1.
(C) The level of MPK6 protein was determined by immunoblot analysis using Ab6NT1.

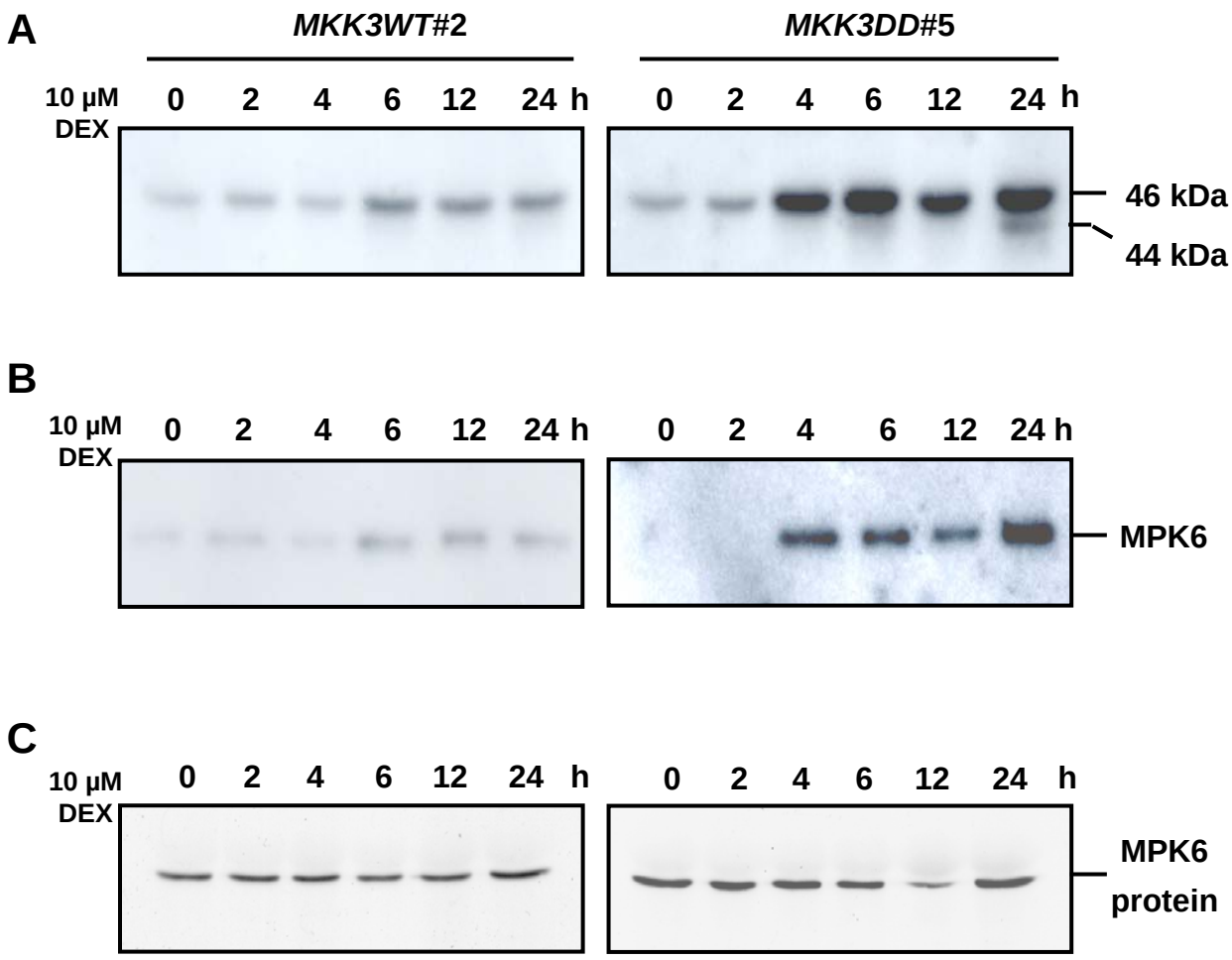

Figure 8. Enhanced expression of *MKK3DD* activates MPK6 *in vivo*.
Induction of *MKK3DD* expression by DEX activates endogenous MPK6.
(A) The activation of endogenous 46 kDa proteins was determined by in-gel kinase assay.
(B) The activation of endogenous MPK6 was determined by in-gel kinase assay using Ab6NT1.
(C) The level of MPK6 protein was determined by immunoblot analysis using Ab6NT1.

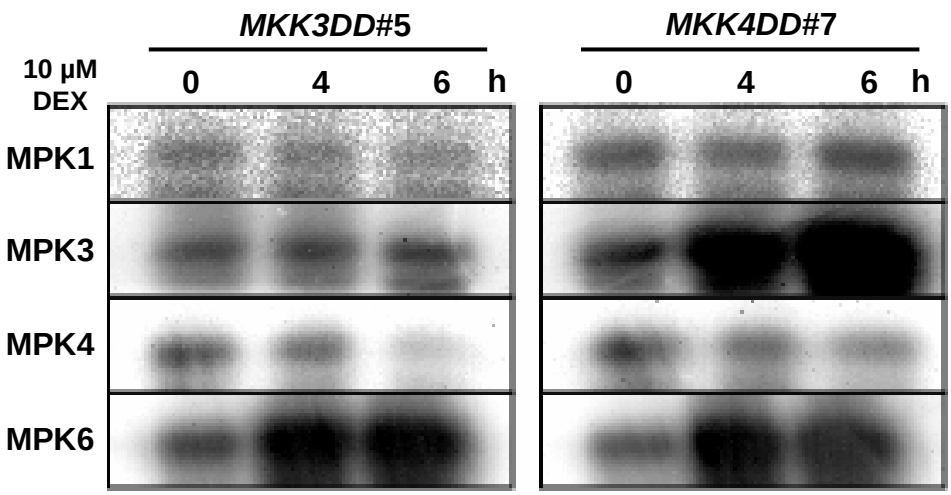

Figure 9. MBP kinase activity of MPK1, MPK3, MPK4, and MPK6 in *MKK3DD* and *MKK4DD* plants.
The *MKK3DD*- and *MKK4DD*-induced activation of MPK1, MPK3, MPK4, and MPK6 was determined using an IP-kinase assay. MPK1, MPK3, MPK4, and MPK6 were immunoprecipitated with specific antibodies and their activity was measured using MBP as a substrate.

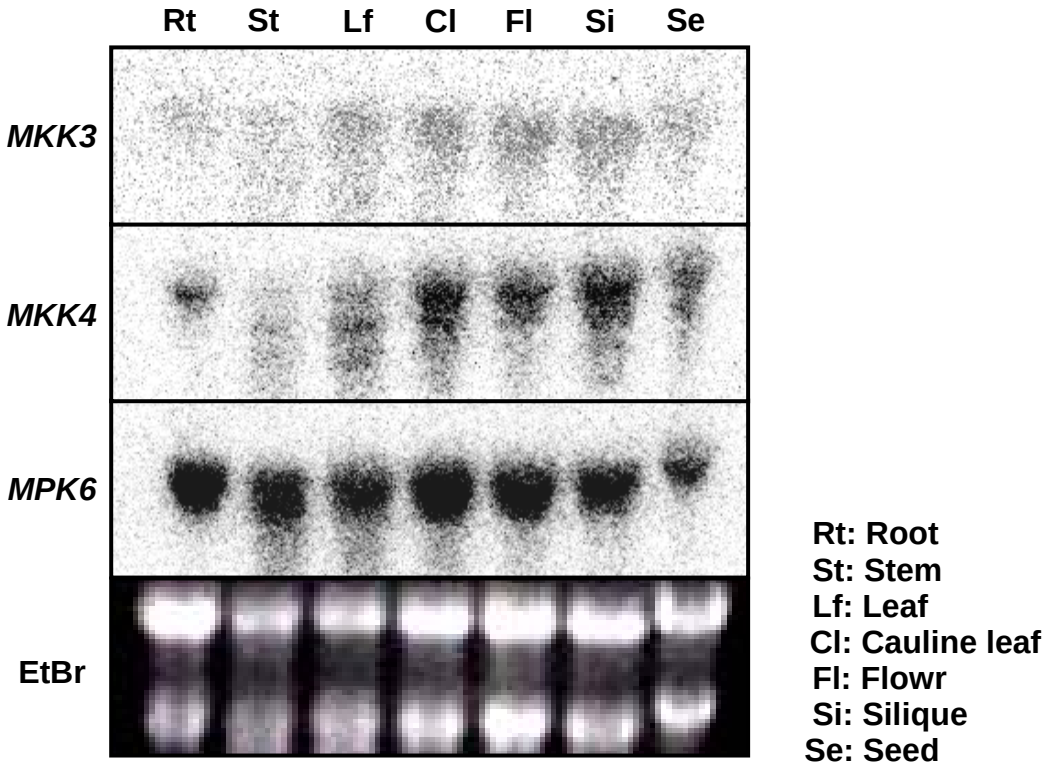

Figure 10. Northern blot analysis of *MKK3*, *MKK4*, and *MPK6* genes in various tissues. Total RNA (20 μg) prepared from root (Rt), stem (St), leaf (Lf), couline leaf (Cl), flower (Fl), silique (Si), and seed (Se) were subjected to electrophoresis. The upper panels show autoradiograph hybridized with the ^{32}P-labeled *MKK3*, *MKK4*, and *MPK6* cDNA. The lower panel shows total RNAs stained with ethidium bromide (EtBr).

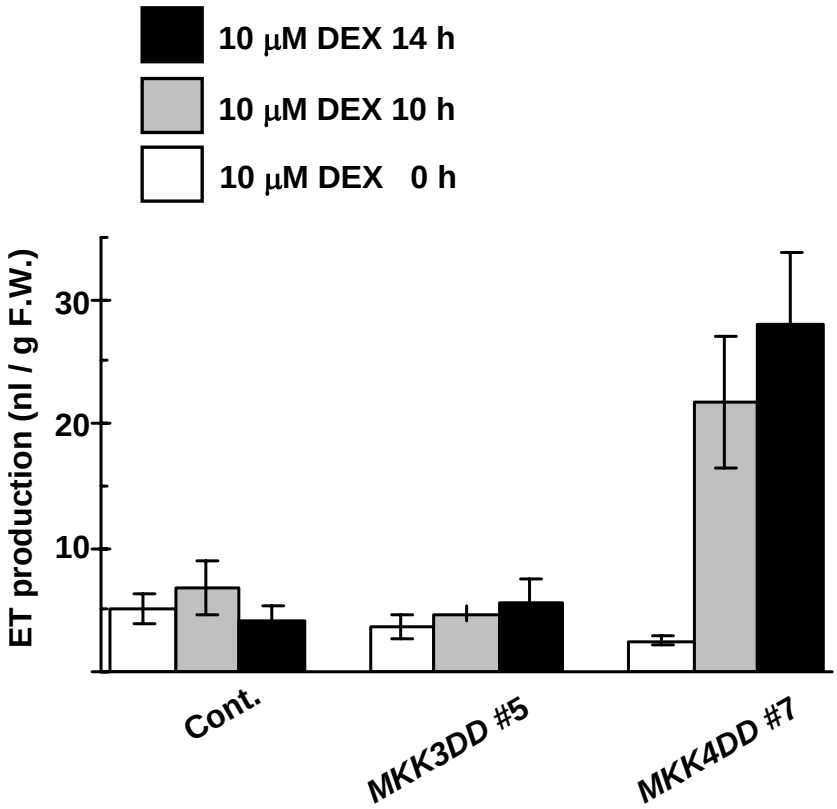

Figure 11. ET production in steroid-inducible *MKK3DD* and *MKK4DD* plants.
The 14-day-old seedlings grown without DEX were transferred to 10 μM DEX solution for indicated time. ET accumulation was measured at indicated times. Values are the mean ± standard deviation of three measurements (each with triplicate samples).

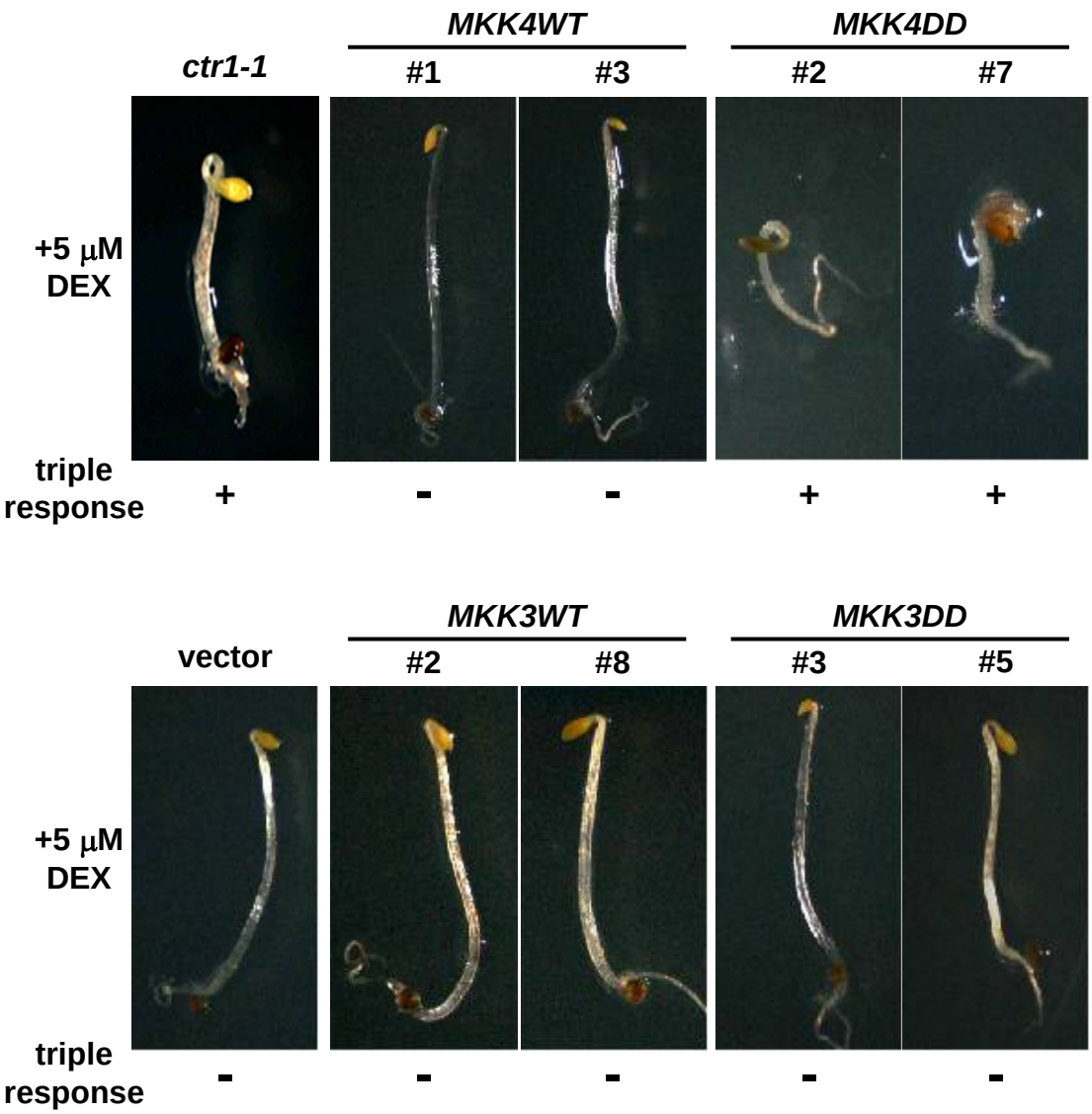

Figure 12. Phenotype observed in steroid-inducible *MKK3DD* and *MKK4DD* transgenic plants. Each transgenic and *ctr1-1* plants was spreaded at the MS plates containing 5 μM DEX, and grown under the dark condition for 3 days.

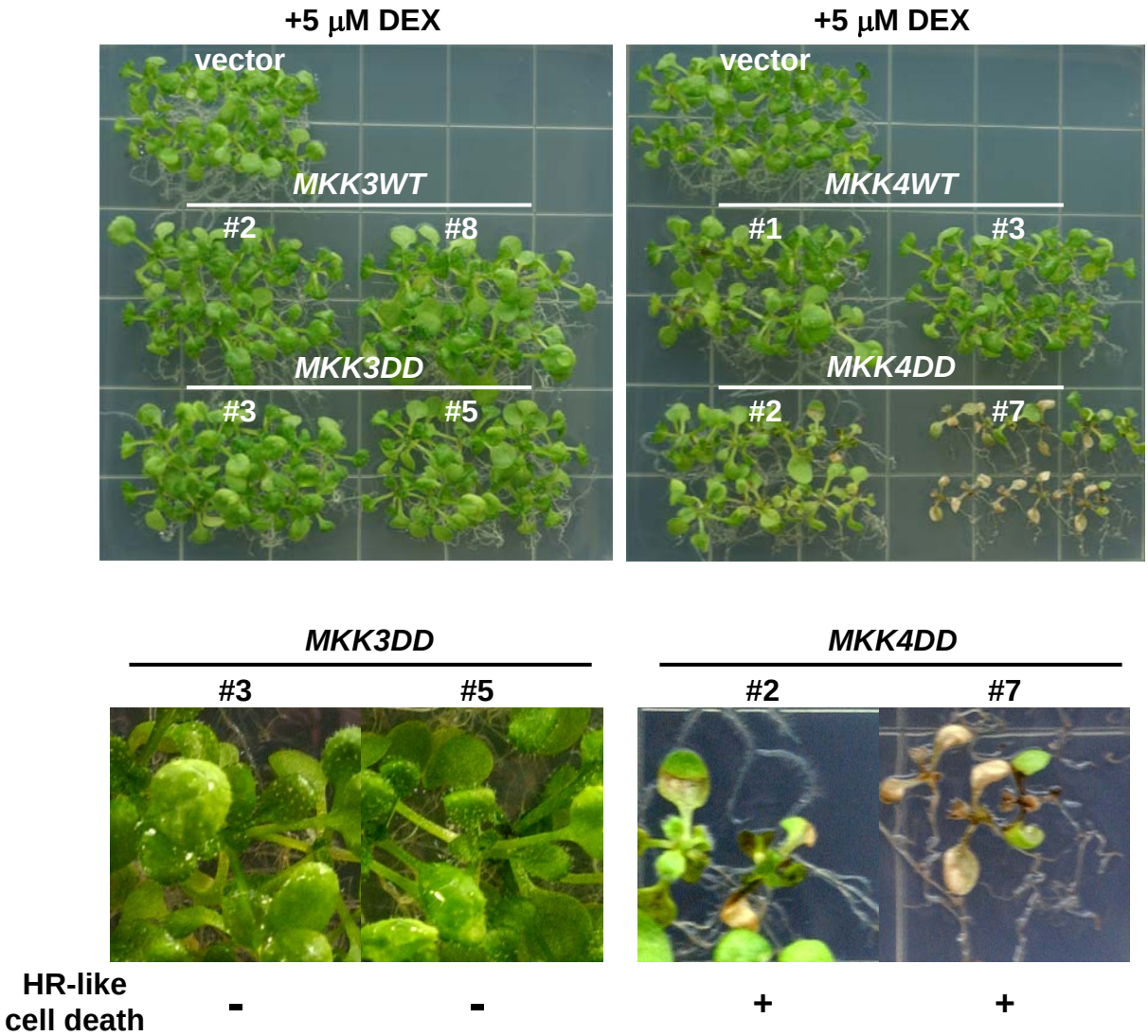

Figure 13. Phenotypic analysis of steroid-inducible transgenic plants.
Phenotype observed in steroid-induced *MKK3DD* and *MKK4DD* transgenic plants. The 7-day-old seedlings grown without DEX were transferred to agar plates containing 5 μM DEX. These pictures were taken after 5 days of transfer. *MKK4DD* plants showed HR-like cell death phenotype. However, any changes were observed in *MKK3DD* plants.

Figure 14. H_2O_2 generation in steroid-inducible *MKK3DD* and *MKK4DD* plants.
The 7-day-old seedlings grown without DEX were transferred to agar plats containing 5 μM DEX for 5 days. H_2O_2 production was detected by the polymerization of DAB.

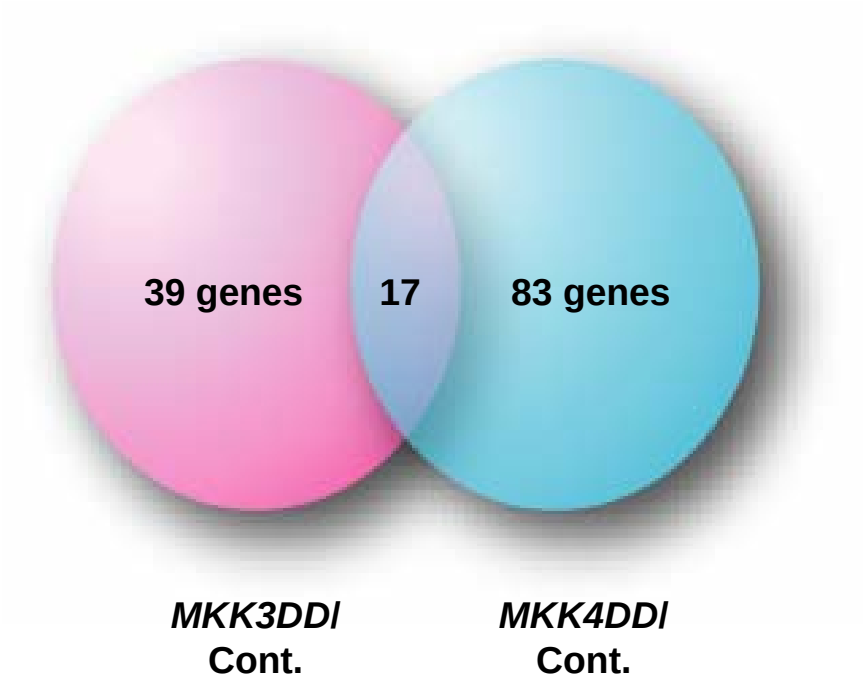

Figure 15. Gene profiling analysis of steroid-inducible *MKK3DD* and *MKK4DD* transgenic plants. Venn diagrams that show at least > or = 5-fold expression difference from control plants. Total RNAs extracted from seedlings which were treated with DEX (10 μM) for 4 h were subjected to microarray analysis.

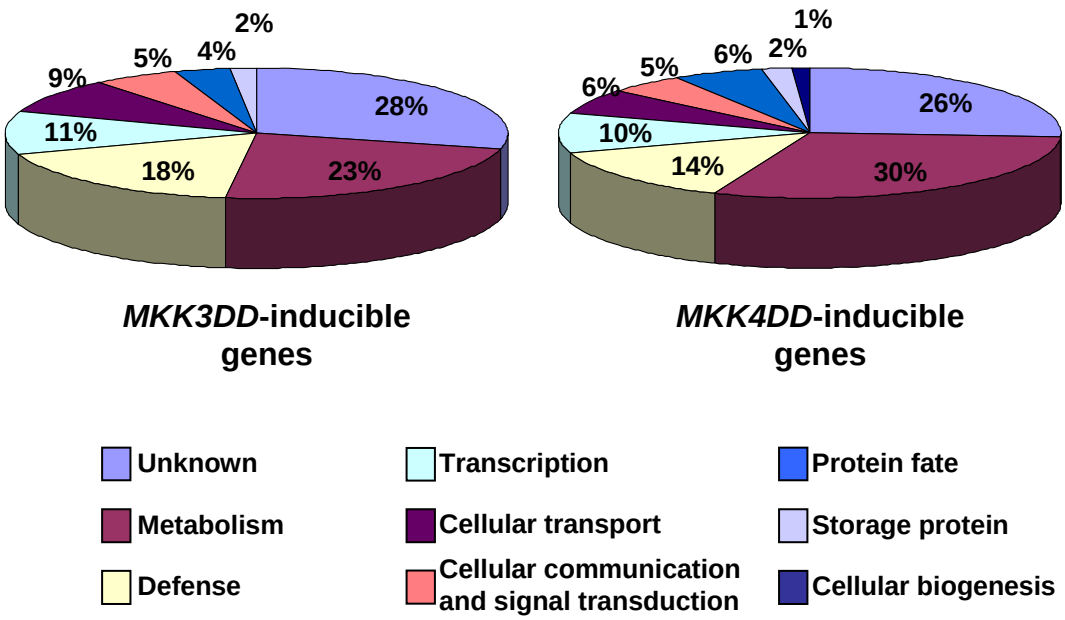

Figure 16. Comparison of predicted functional distribution of *MKK3DD*- and *MKK4DD*-expressed genes.
Upregulated genes were classified according to their putative functions based on the classification of the Munich Information Center for Protein Sequence (MIPS) database, and determined whether MKK3 and MKK4 pathway allocate gene expression patterns differently among classifications.

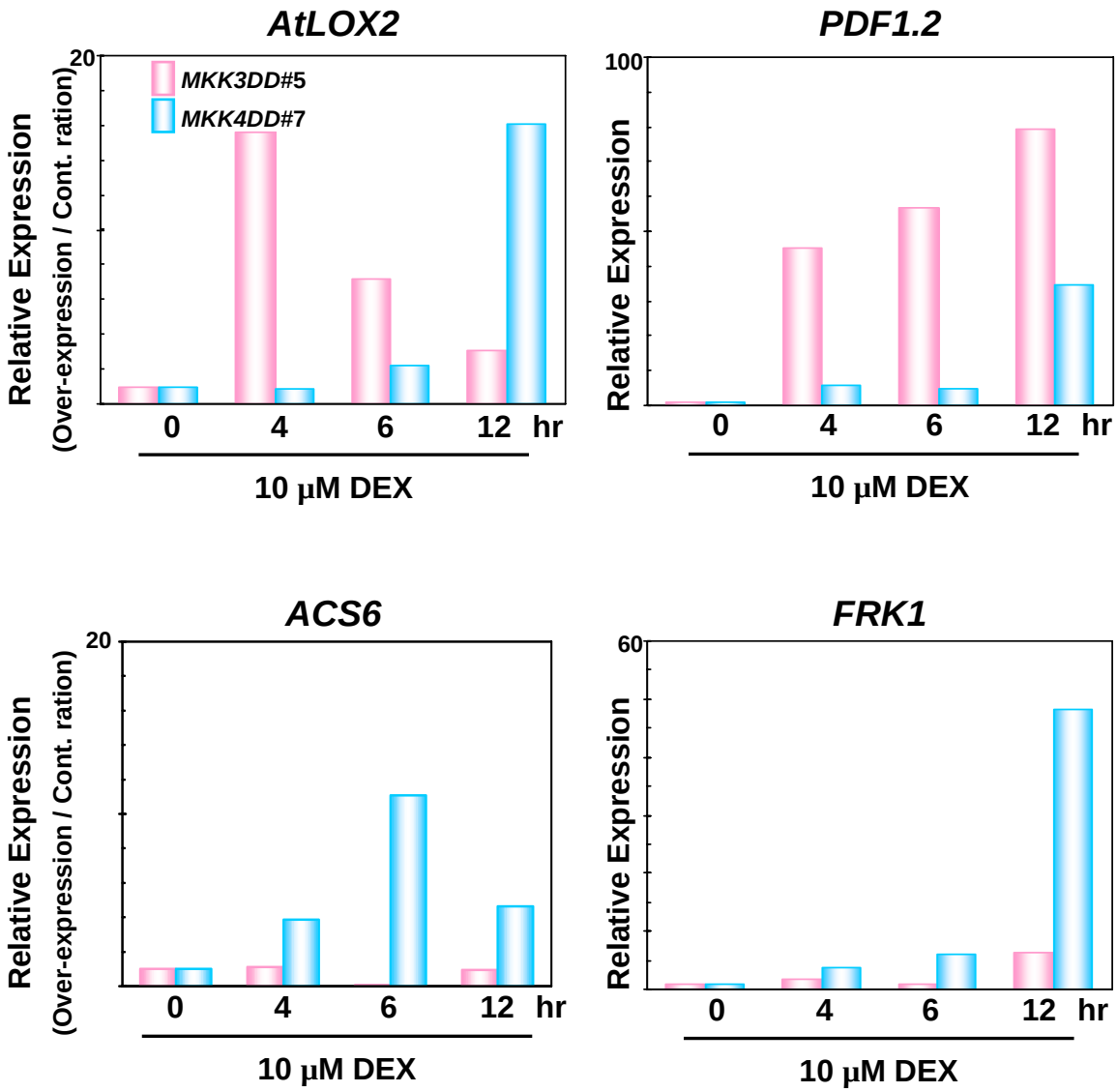

Figure 17. Relative expression of wounding- and pathogen-inducible genes in *MKK3DD* and *MKK4DD* plants.

The 14-day-old seedlings grown without DEX were transferred to 10 μM DEX solution for indicated time. Their total RNA was isolated, and subjected to QRT-PCR analysis. The transgenic plants carrying empty vector were used as a control. The transcript levels of *AtLOX2*, *PDF1.2*, *ACS6*, and *FRK1* were normalized to the expression of *β-actin* measured in the same RNA samples. Pink bar represents *MKK3DD* plants, and blue bar represents *MKK4DD* plants. Data are the mean ± standard deviation of two independent experiments.

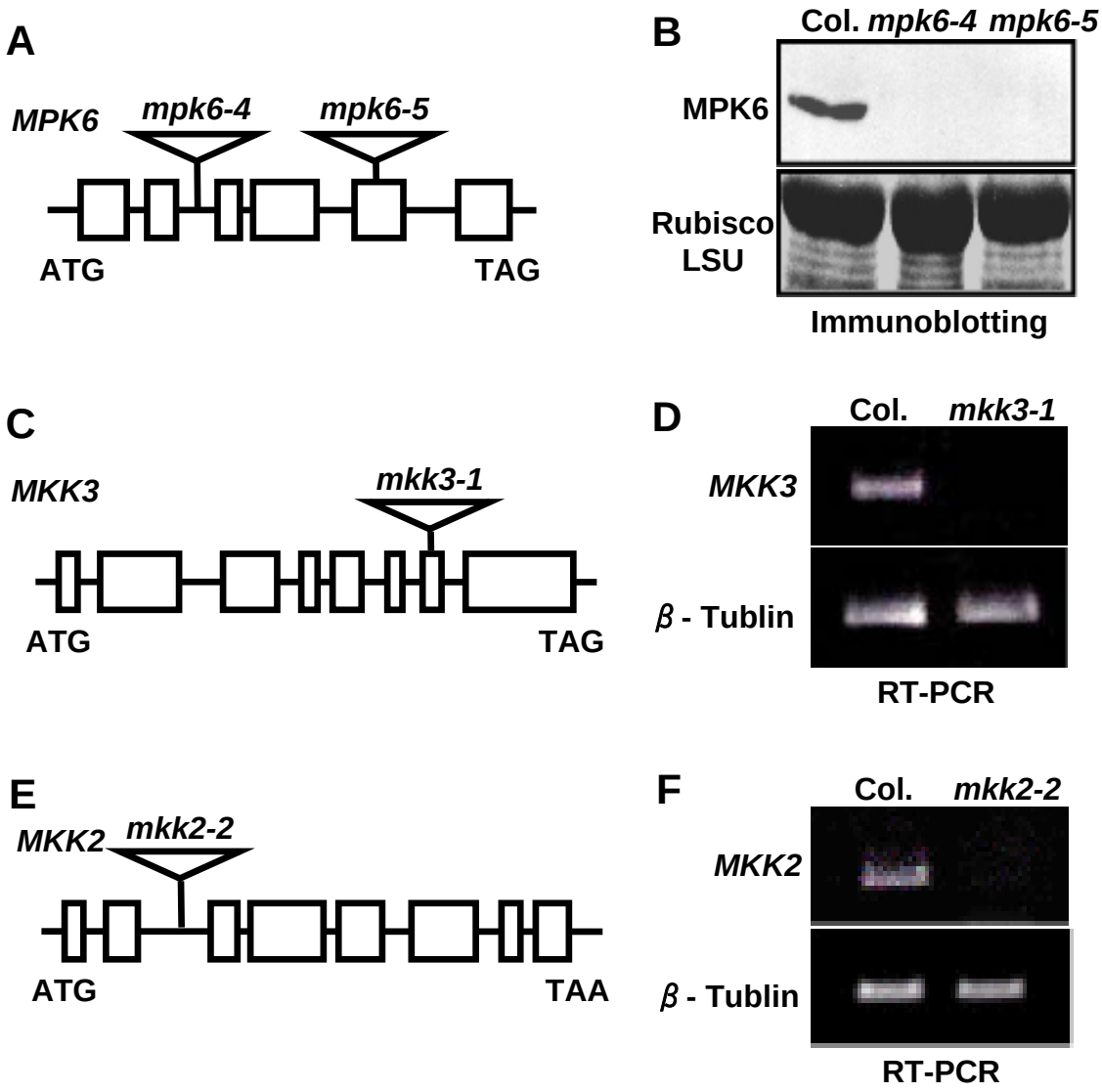

Figure 18. Isolation of T-DNA insertion mutants of *MKK2, MKK3, MPK6* genes.
(A) T-DNA knockout mutants of *MPK6*. Exons are indicated as white boxes. T-DNA is inserted in the second intron (*mpk6-4*) and the fifth exon (*mpk6-5*), respectively.
(B) Immunoblot analysis of MPK6 in wild-type and *mpk6* plants.
(C) T-DNA knockout mutants of *MKK3*. Exons are indicated as white boxes. T-DNA is inserted in the seventh exon.
(D) RT-PCR analysis of *MKK3* in wild-type and *mkk3-1* plants.
(E) T-DNA knockout mutants of *MKK2*. Exons are indicated as white boxes. T-DNA is inserted in the second intron.
(F) RT-PCR analysis of *MKK2* in wild-type and *mkk2-2* plants.

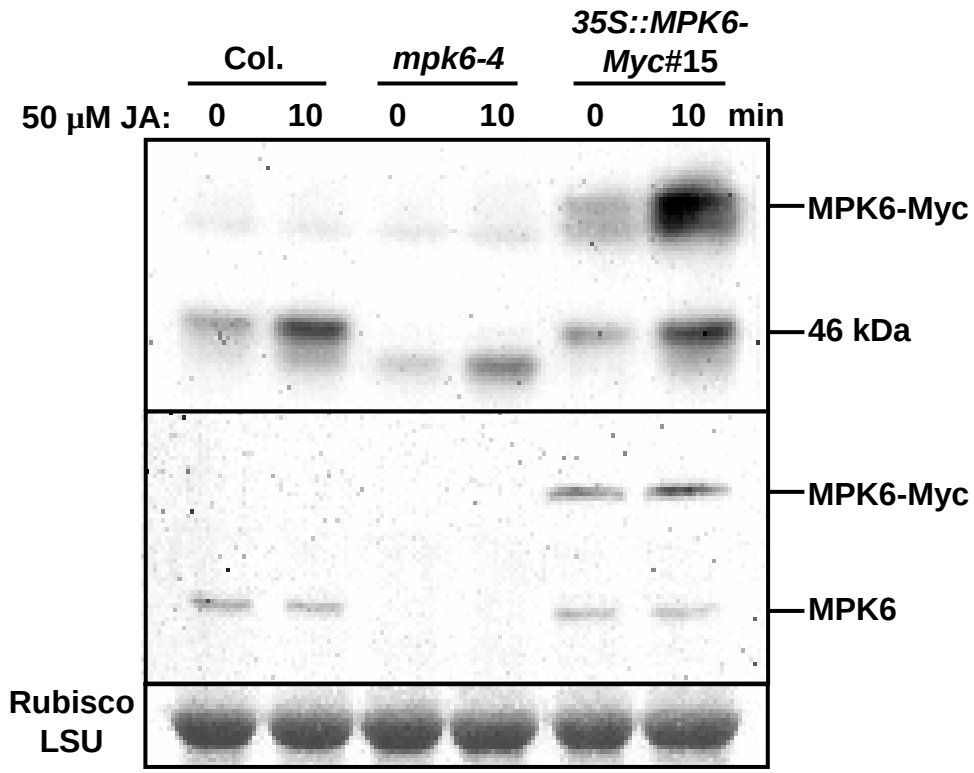

Figure 19. JA-dependent kinase activation of MPK6.
Wild-type, *mpk6-4*, and *35S::MPK6-Myc*#15 plants were treated with 50 µM JA for 10 min, and their protein extracts were subjected to an in-gel kinase assay with MBP as a substrate (top). Immunoblot analysis of MPK6 (middle) and the large subunit of Rubisco LSU stained by CBB are shown as additional control for equal protein amounts (bottom).

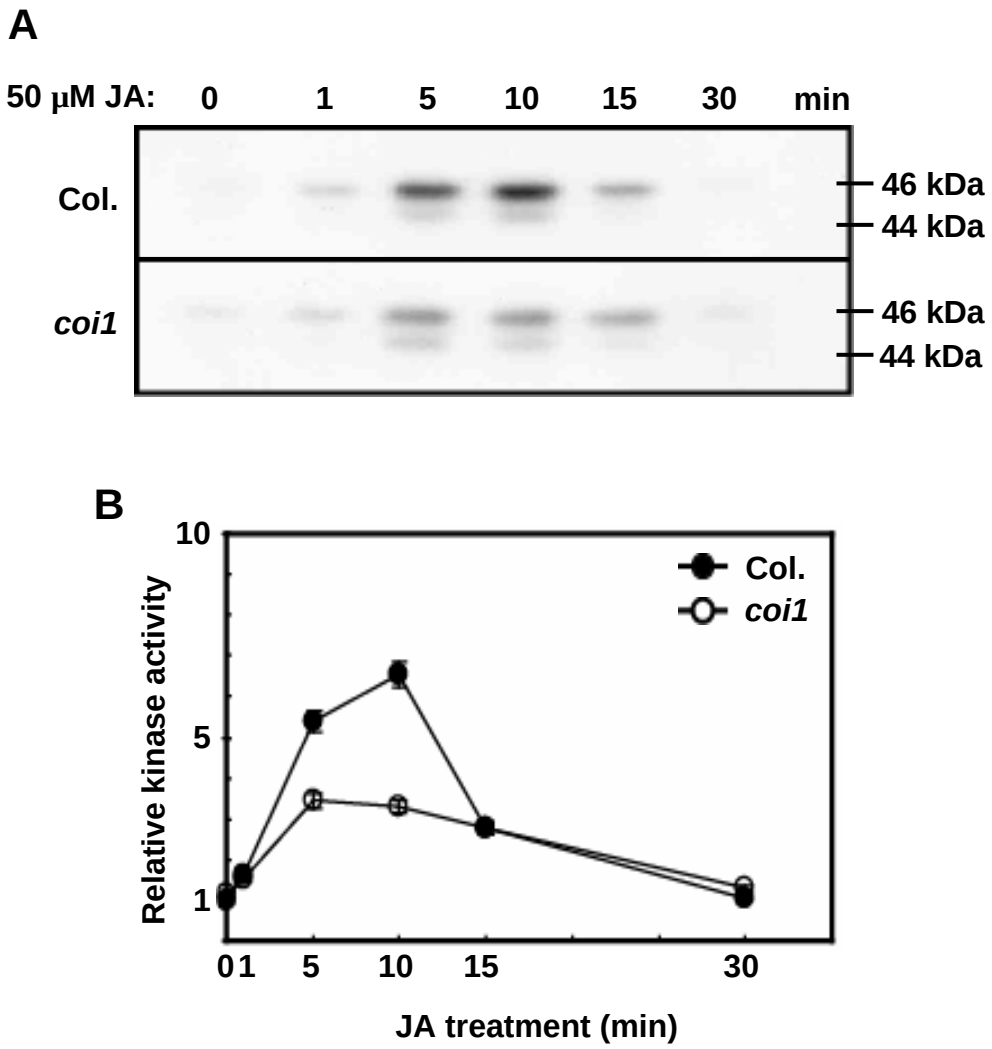

Figure 20. The kinase activation of MPK6 affected by *coi1* mutation.
(A) JA-dependent 46-kDa kinase activity in *coi1* mutant. Wild-type and *coi1* plants were treated with 50 μM JA for indicated times, and their protein extracts were subjected to an in-gel kinase assay with MBP as a substrate.
(B) Relative 46 kDa kinase activities in wild-type and *coi1* plants in response to 50 μM JA.

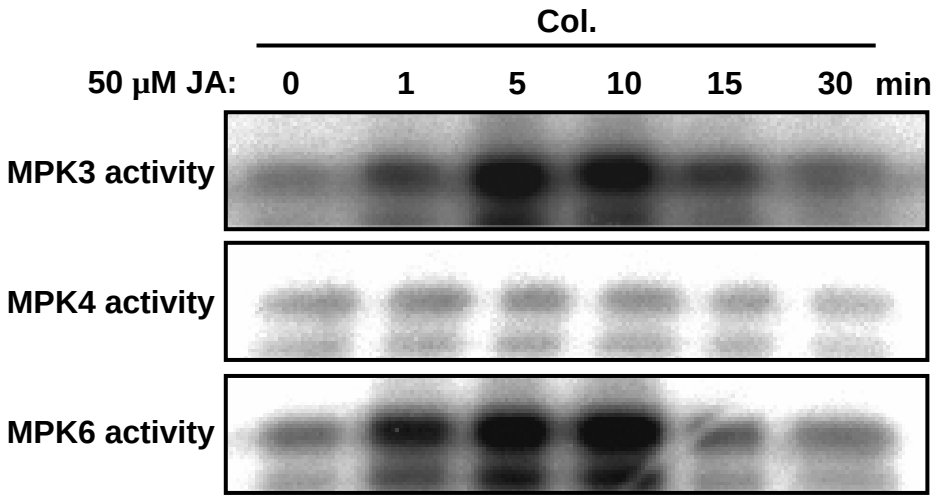

Figure 21. Effect of JA on the activation of MPK3, MPK4 and MPK6 in wild-type plants. MBP kinase activity of MPK4 and MPK6 were determined by IP-kinase assay. MPK3, MPK4, and MPK6 were immunoprecipitated with specific antibodies and their activity was measured in tube using MBP as a substrate.

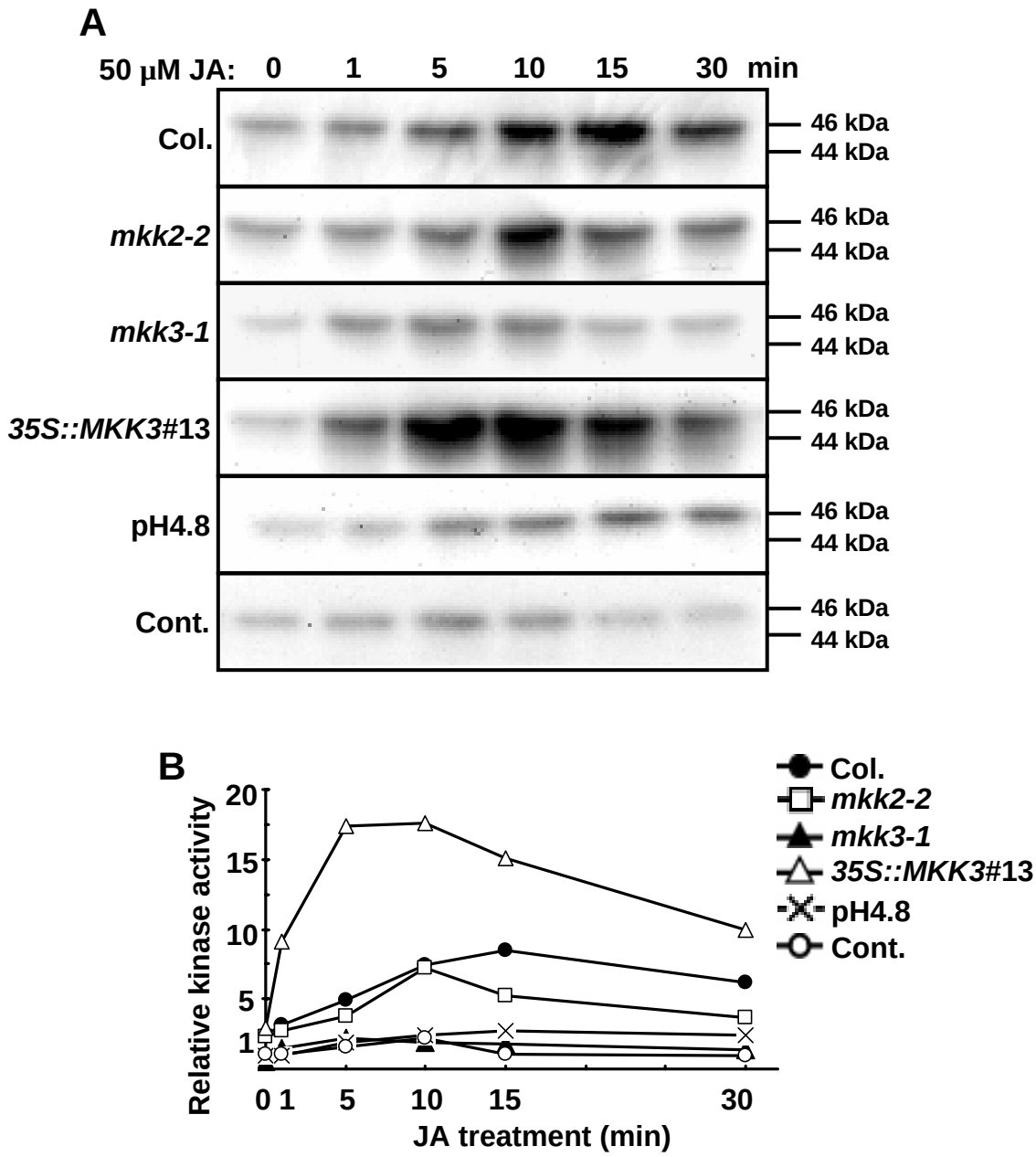

Figure 22. Effects of MKK3 on the JA-dependent activation of MPK6.
(A) Wild-type, *mkk2-2*, *mkk3-1*, and *35S::MKK3*#13 plants were treated with 50 μM JA for indicated times, and their protein extracts were subjected to an in-gel kinase assay with MBP as a substrate. As a control, wild-type plants were treated with 0.012% ethanol adjusted to a pH of 4.8 (pH4.8) or unadjusted (Cont.).
(B) Relative 46-kDa kinase activities in wild-type, *mkk2-2*, *mkk3-1*, *35S::MKK3*#13, pH4.8, and control plants.

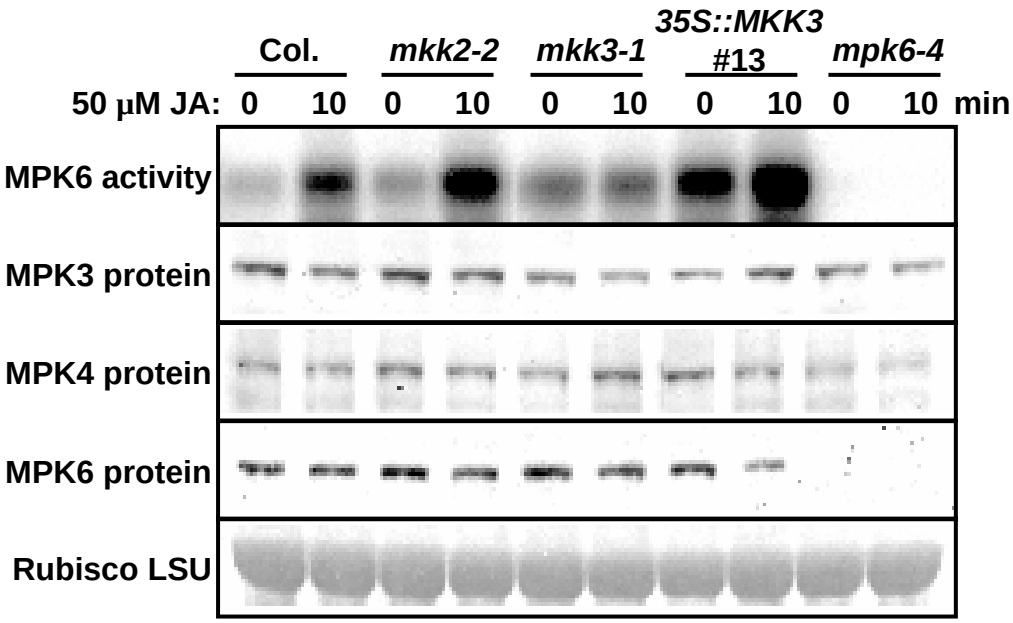

Figure 23. Effect of JA on the activation of MPK6 and immunoblot analysis of MPK3, MPK4, and MPK6 proteins in wild-type, *mkk2-2*, *mkk3-1*, 35S::*MKK3*#13, and *mpk6-4* plants.
Samples were taken 0 and 10 min after JA treatment and used for IP-kinase assay and immunoblot analysis. MPK6 was immunoprecipitated with specific antibodies and the activity was measured in tube using MBP as a substrate. MPK3, MPK4, MPK6 protein levels were determined by immunoblot analysis. Equal protein loading was confirmed by staining the LSU with CBB**.**

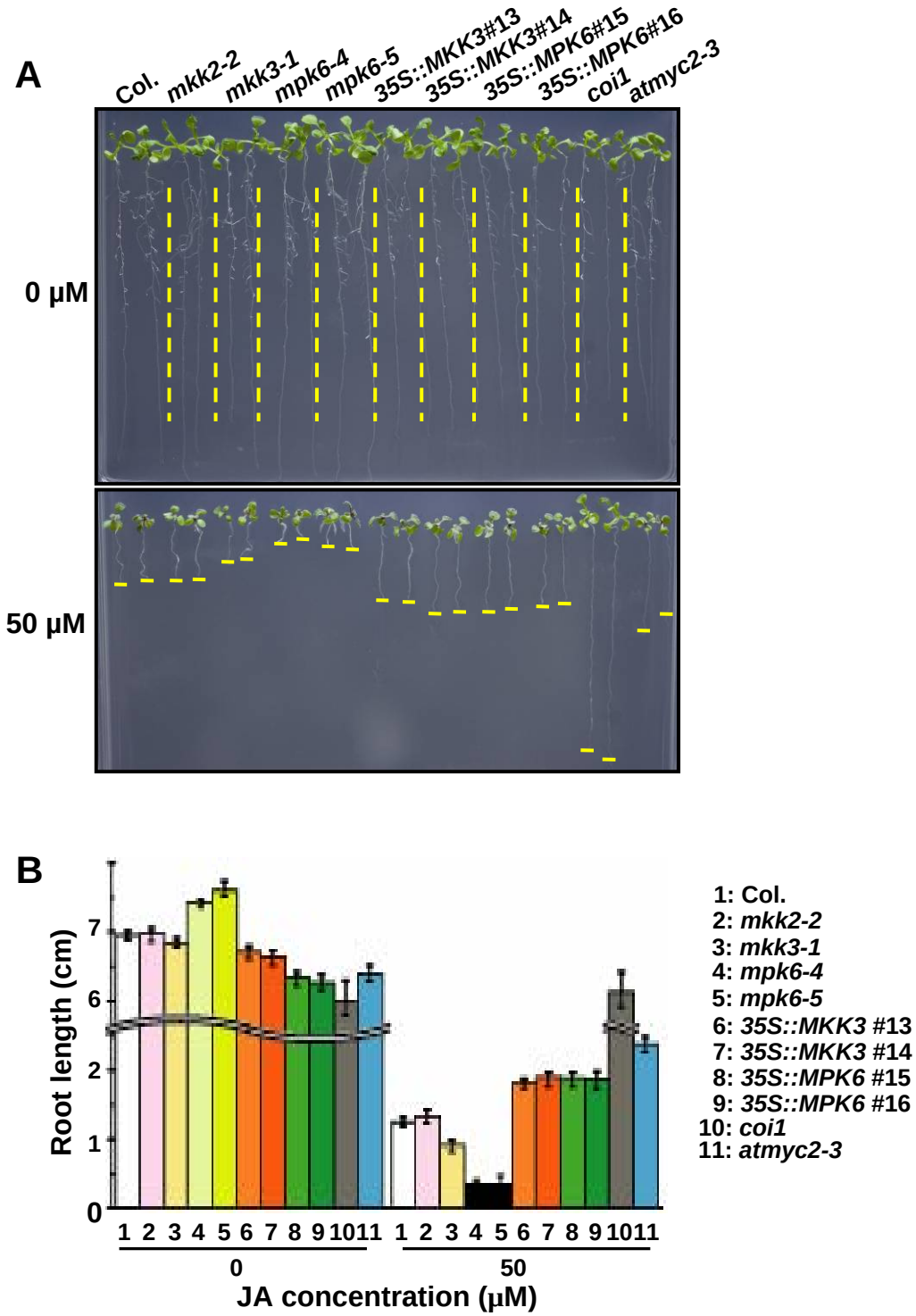

Figure 24. MKK3 and MPK6 affect JA-dependent root growth sensitivity.
(A) Effect of JA on the inhibition of root growth in wild-type (lane *1*), *mkk2-2* (lane *2*), *mkk3-1* (lane *3*), *mpk6-4* (lane *4*), *mpk6-5* (lane *5*), *35S::MKK3*#13 (lane *6*), *35S::MKK3*#14 (lane *7*), *35S::MPK6-Myc*#15 (lane *8*), *35S::MPK6-Myc*#16 (lane *9*), *coi1* (lane *10*), and *atmyc2-3* plants (lane *11*). Seedlings were grown on MS plates with or without 50 mM JA, and these pictures were taken after a 10-day growth period.
(B) Degree of JA-dependent root growth inhibition in each genotype. Root length of these plants was measured after the 10-day growth period. Root growth, in terms of length, was calculated from the results of three independent experiments (n = 8 each). Bars indicate standard deviations.

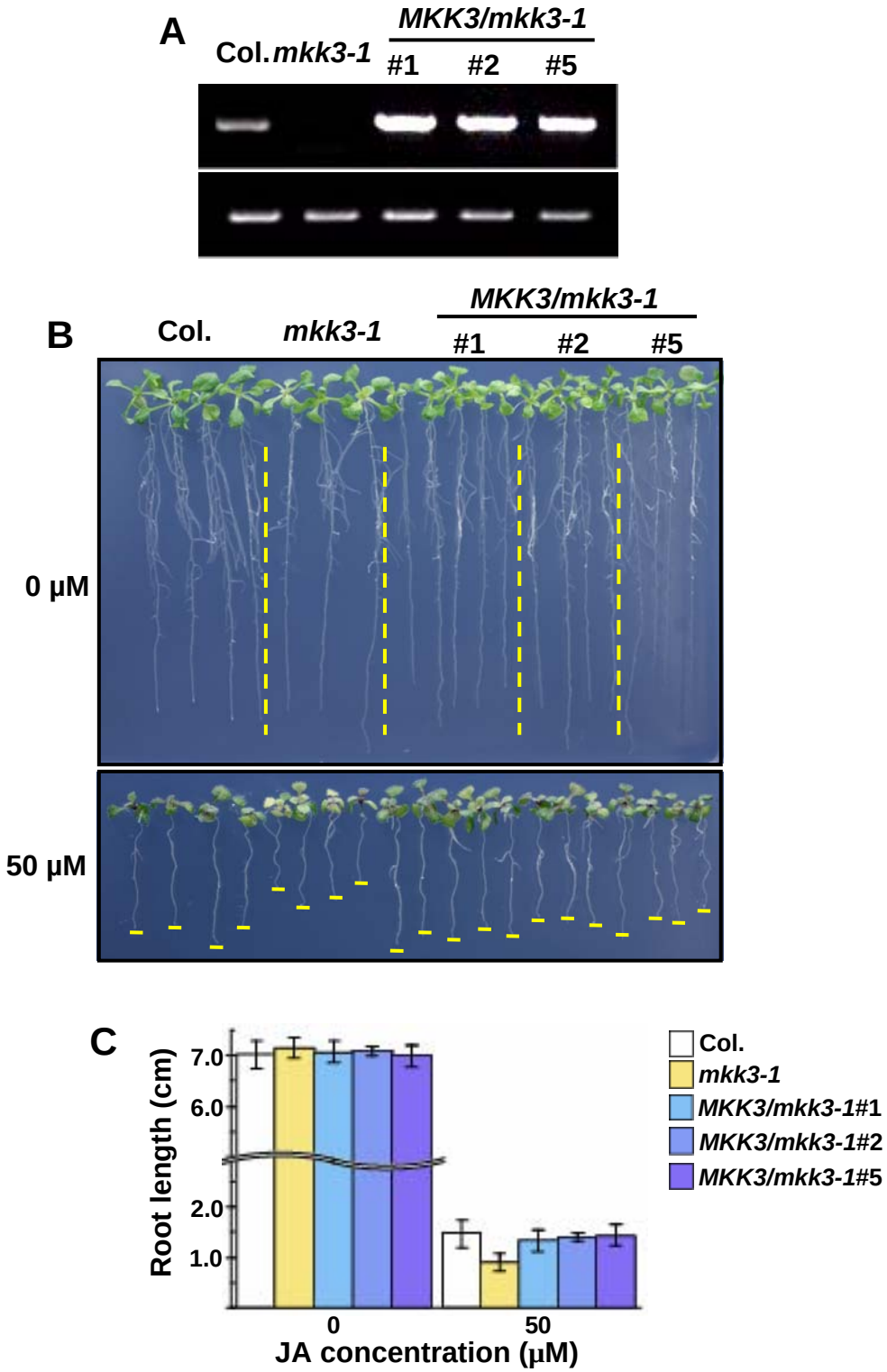

Figure 25. Complementation of the JA-sensitive phenotype of *mkk3-1* by 35S::*MKK3*.
(A) RT-PCR analysis of *MKK3* expression in *MKK3*/*mkk3-1* plants. Three independent lines (lines #1, #2, and #5) were used for the phenotypic analysis.
(B) Effect of JA on the inhibition of root growth in wild-type, *mkk3-1*, and *MKK3*/*mkk3-1* plants. Seedlings were grown on MS plates with or without 50 mM JA; these photographs were taken after 10 days of growth.
(C) Root lengths were measured (n = 8) and root growth was calculated from the results of three independent experiments. Mean ± standard deviation is shown.

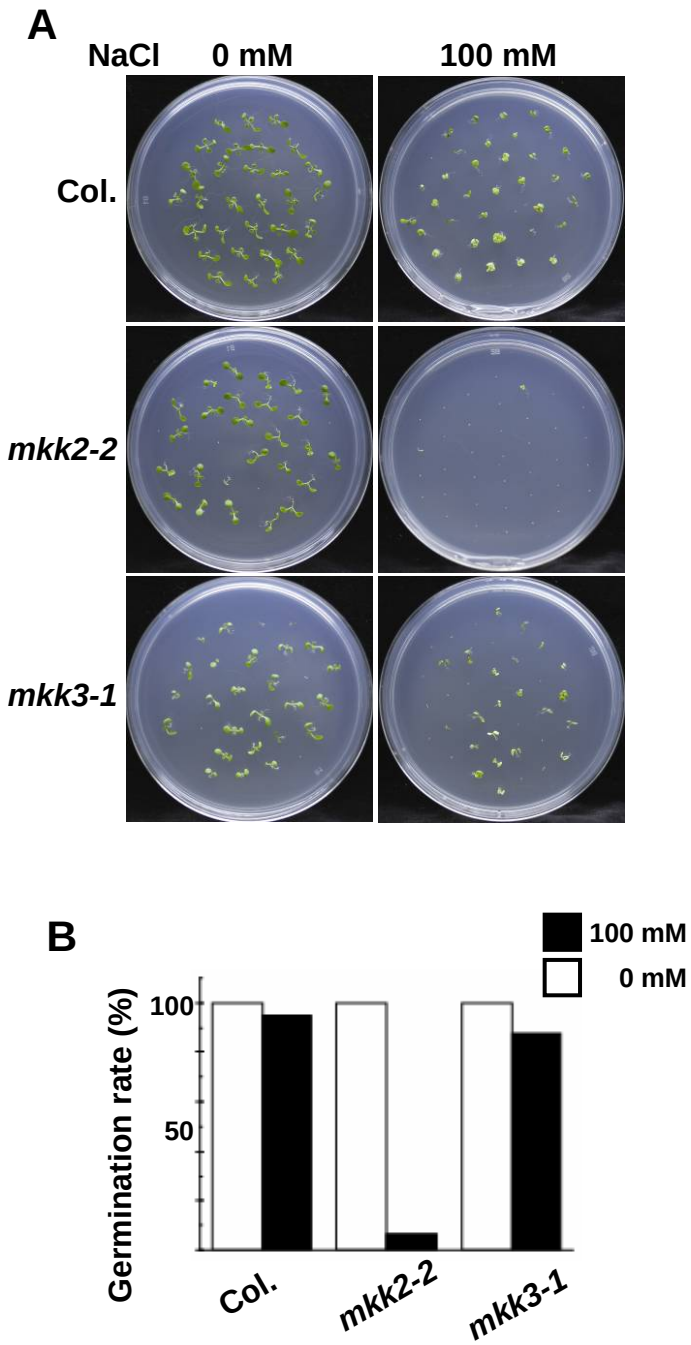

Figure 26. Effect of salt on the germination of MKK-null mutants.
(A) Seedlings of wild-type, *mkk2-2*, and *mkk3-1* mutant were grown on MS plates with (black bar) or without (white bar) 100 mM NaCl, and these photos were taken after a 10-day growth period.
(B) Germination rates in each genotype. White and black bar represent 0 mM and 100 mM NaCl, respectively.

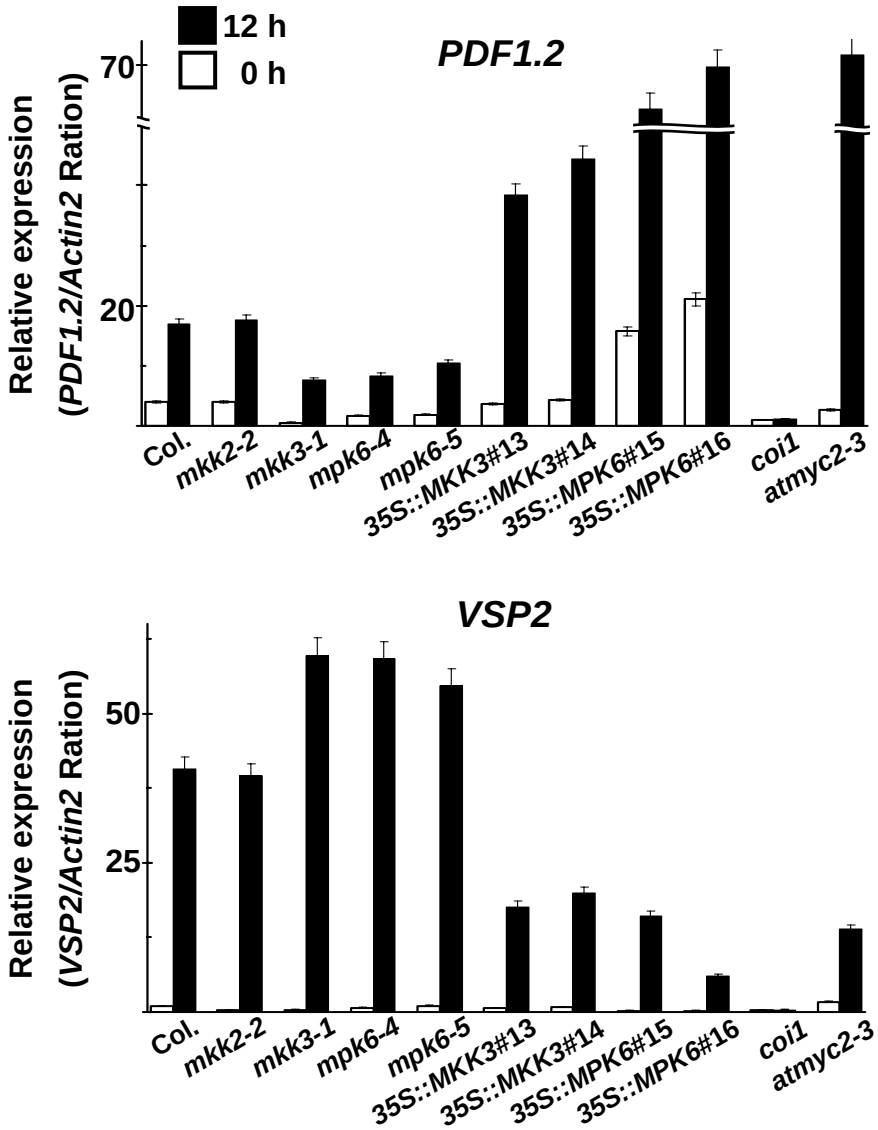

Figure 27. The MKK3-MPK6 cascade regulates the expression of *PDF1.2* and *VSP2* genes in the JA signaling.

The relative transcription levels of *PDF1.2* and *VSP2* in wild-type, *mkk2-2*, *mkk3-1*, *mpk6-4*, *mpk6-5*, *35S::MKK3* (lines 13 and 14), *35S::MPK6-Myc* (lines 15 and 16), *coi1*, and *atmyc2-3* plants. Total RNA was isolated from non-treated seedlings (white bar) or seedlings treated with 50 μM JA for 12 h (black bar), and subjected to QRT-PCR analysis. The *PDF1.2* and *VSP2* transcript levels were normalized to the expression of *β-actin* measured in the same RNA samples. Data are the mean ± standard deviation of two independent experiments.

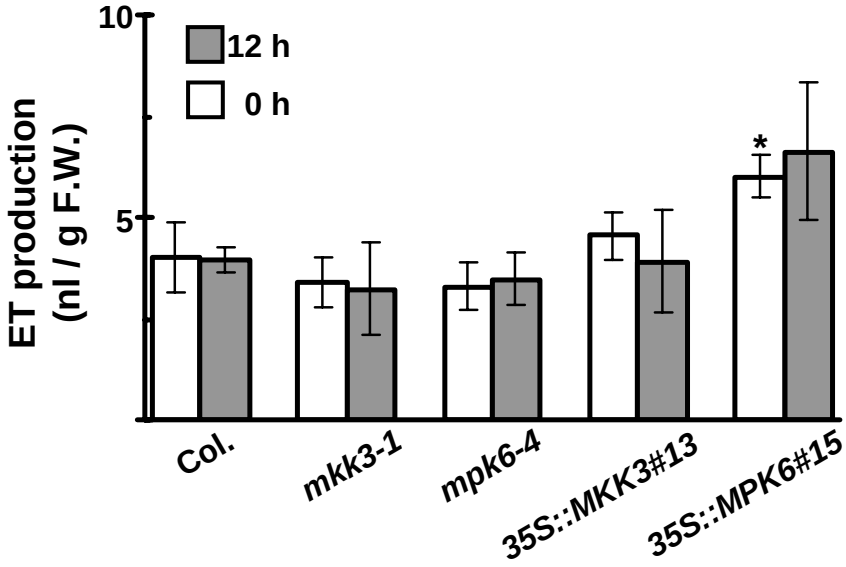

Figure 28. The accumulation of ET production by JA-activated MKK3-MPK6 cascade. ET production in wild-type, *mkk3-1*, *mpk6-4*, *35S::MKK3*#13, and *35S::MPK6-Myc*#15 plants. The 14-day-old seedlings were treated with (gray bar) or without (white bar) 50 μM JA for 12 h. ET accumulation was measured at the indicated times. Values shown are the mean ± standard deviation of three measurements (each with triplicate samples). Asterisks represent significant difference between non-treated wild-type and *35S::MPK6-Myc*#15 plants (unpaired *t* test: * $P < 0.01$).

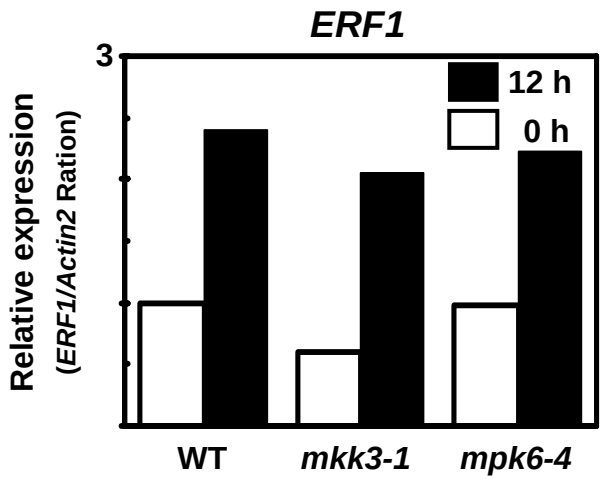

Figure 29. The relative expression level of *ERF1* gene regulated by JA-activated MKK3-MPK6 cascade.

The relative transcription levels of *ERF1* in wild-type, *mkk3-1*, and *mpk6-4* plants. Total RNAs were isolated from non-treated seedlings (white bar) or seedlings treated with 50 μM JA for 12 hours (black bar), and subjected to QRT-PCR analysis. The *ERF1* transcript levels were normalized to the expression of *β-actin* measured in the same RNA samples.

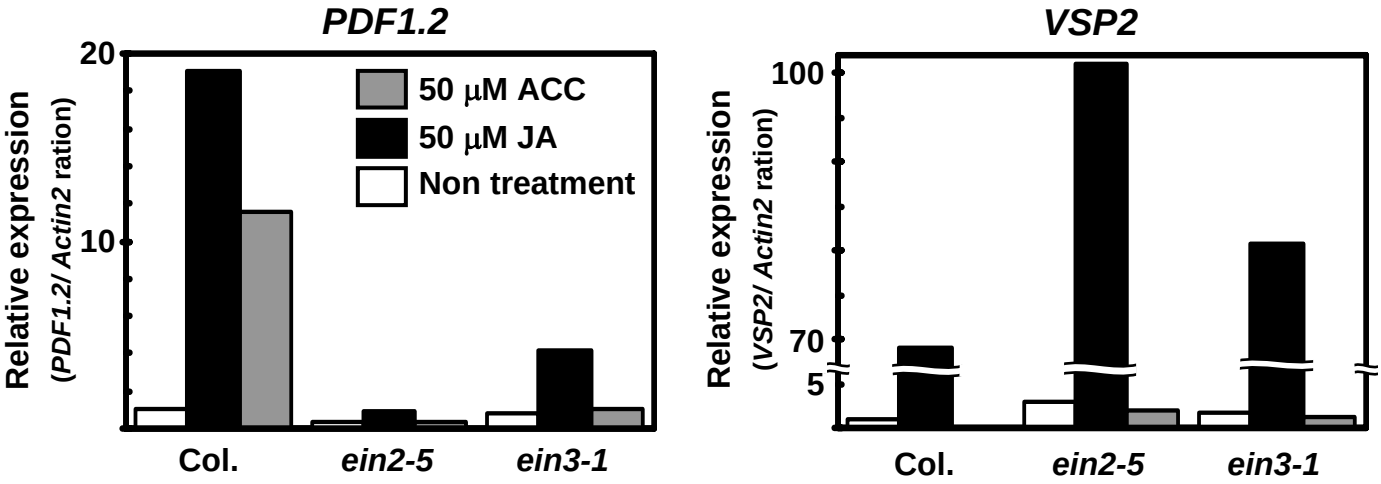

Figure 30. Analysis of expression from JA-regulated genes in *ein2* and *ein3* mutants. Quantification of the relative abundance of the *PDF1.2* and *VSP2* transcripts in non- or 50 μM JA- or 50 μM ACC-treated wild-type (Col.), *ein2-5*, and *ein3-1* mutants. Total RNA was isolated from platns 12 h after each treatment, converted to cDNA, and used as template in QRT-PCR analysis. The *PDF1.2* and *VSP2* transcript levels were normalized to the expression of *β*-actin measured in the same RNA samples.

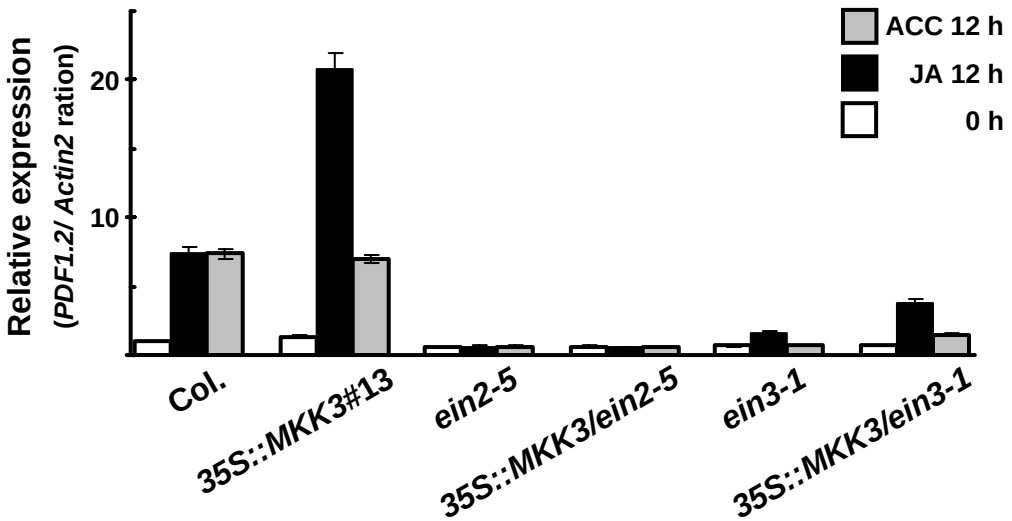

Figure 31. The *PDF1.2* expression concomitantly regulated in MKK3-MPK6 cascade.
Relative transcription levels of *PDF1.2* in JA-treated wild-type, *35S::MKK3*#13, *ein2-5*, *35S::MKK3*/*ein2-5*, *ein3-1*, and *35S::MKK3*/*ein3-1* plants. Total RNA was isolated from untreated seedlings (white bars) or seedlings treated for 12 h with 50 μM JA (black bars) or 50 μM ACC (gray bars) and subjected to QRT-PCR analysis. *PDF1.2* and *VSP2* transcript levels were normalized to the expression of *β-actin* measured in the same samples. Data are the mean ± standard deviation of two independent experiments.

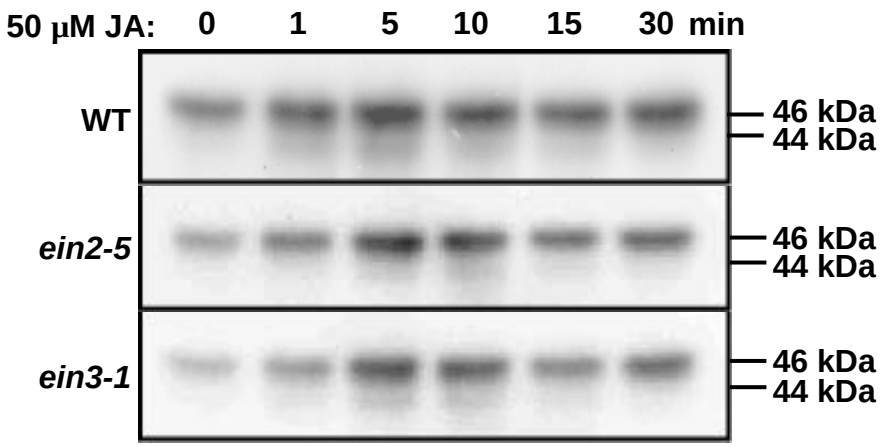

Figure 32. The kinase activation of MPK6 affected by *ein2,* and *ein3* mutation. JA-dependent 46 kDa activities in wild-type, *ein2-5*, and *ein3-1* plants. These plants were treated with 50 μM JA for the indicated times, and their protein extracts were subjected to an in-gel kinase assay with MBP as a substrate.

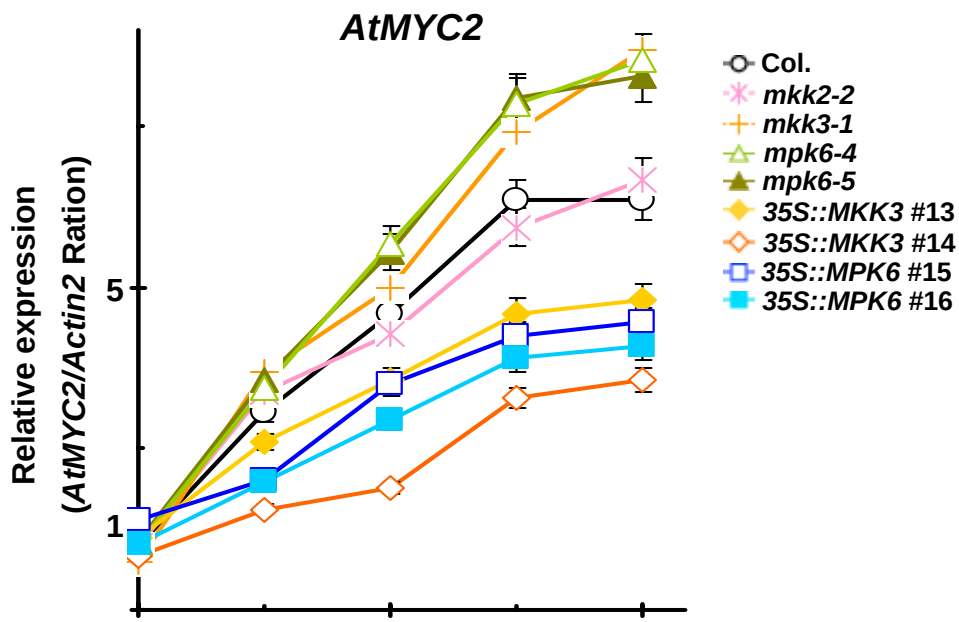

Figure 33. The relative expression level of *AtMYC2* gene regulated by JA-activated MKK3-MPK6 cascade.

The relative transcription levels of *AtMYC2* in wild-type, *mkk2-2*, *mkk3-1*, *mpk6-4*, *mpk6-5*, *35S::MKK3* (lines 13 and 14), and *35S::MPK6-Myc* (lines 15 and 16) plants. Total RNA was isolated from seedlings treated with 50 μM JA for the indicated times and subjected to QRT-PCR analysis. *AtMYC2* transcript levels were normalized to the expression of *β-actin* measured in the same samples. Data are the mean ± standard deviation of two independent experiments.

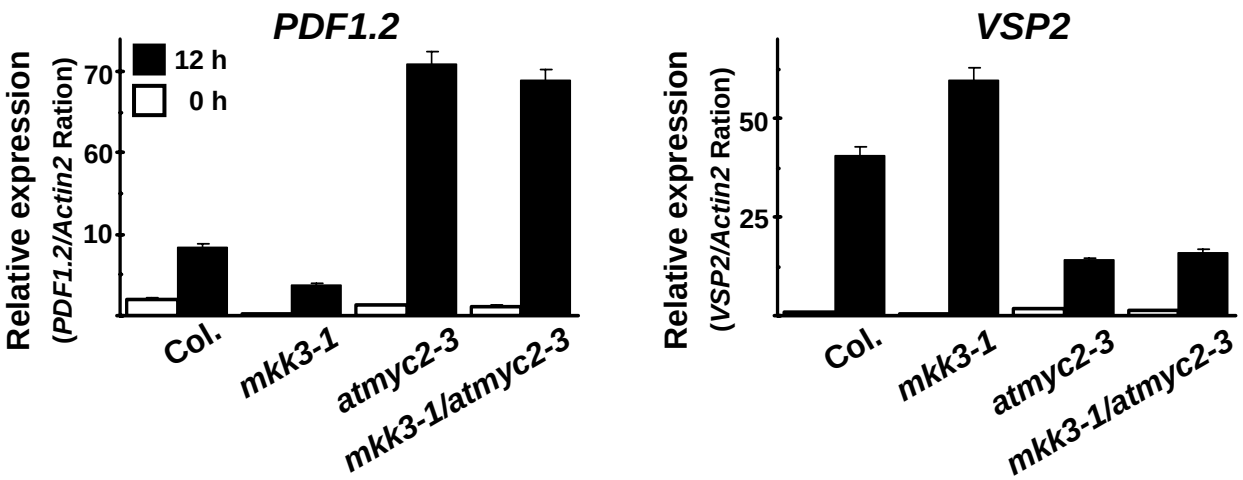

Figure 34. The relative transcription levels of *PDF1.2* and *VSP2* in wild-type, *mkk3-1*, *atmyc2-3*, and *mkk3-1/atmyc2-3* plants.
Total RNA was isolated from untreated seedlings (white bar) or seedlings treated with JA for 12 h (black bar), and subjected to QRT-PCR analysis. The *PDF1.2* and *VSP2* transcript levels were normalized to the expression of *β-actin* measured in the same RNA samples. Data are the mean ± standard deviation of two independent experiments.

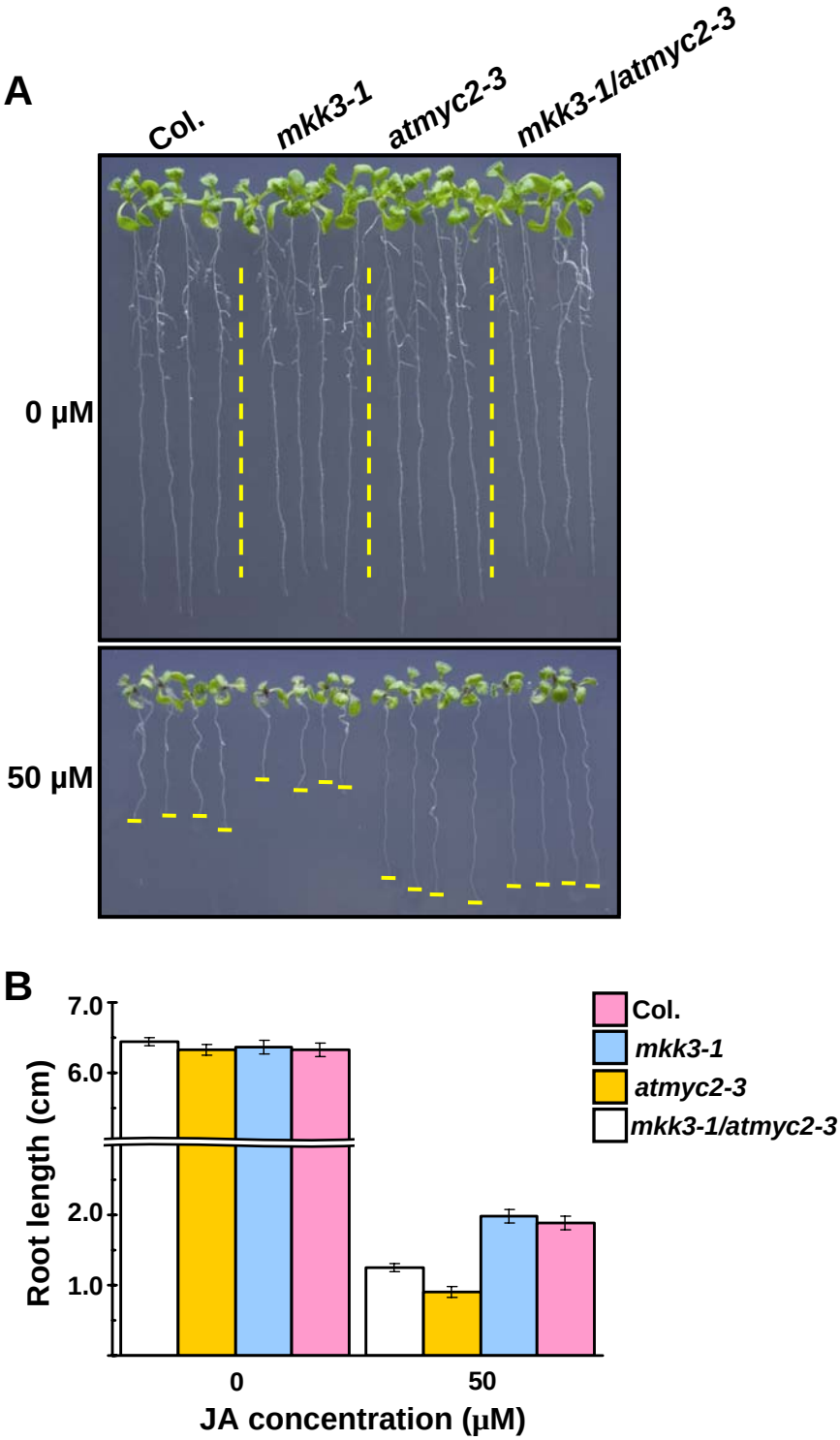

Figure 35. Effect of JA on the inhibition of root growth in wild-type, *mkk3-1*, *atmyc2-3*, and *mkk3-1/atmyc2-3* plants.
(A) Each seedling was grown on MS plates with 50 μM JA. These photos were taken after a 10-day growth period.
(B) Root growth, in terms of length, was calculated from the results of three independent experiments (n = 8 each). Bars indicate standard deviations.

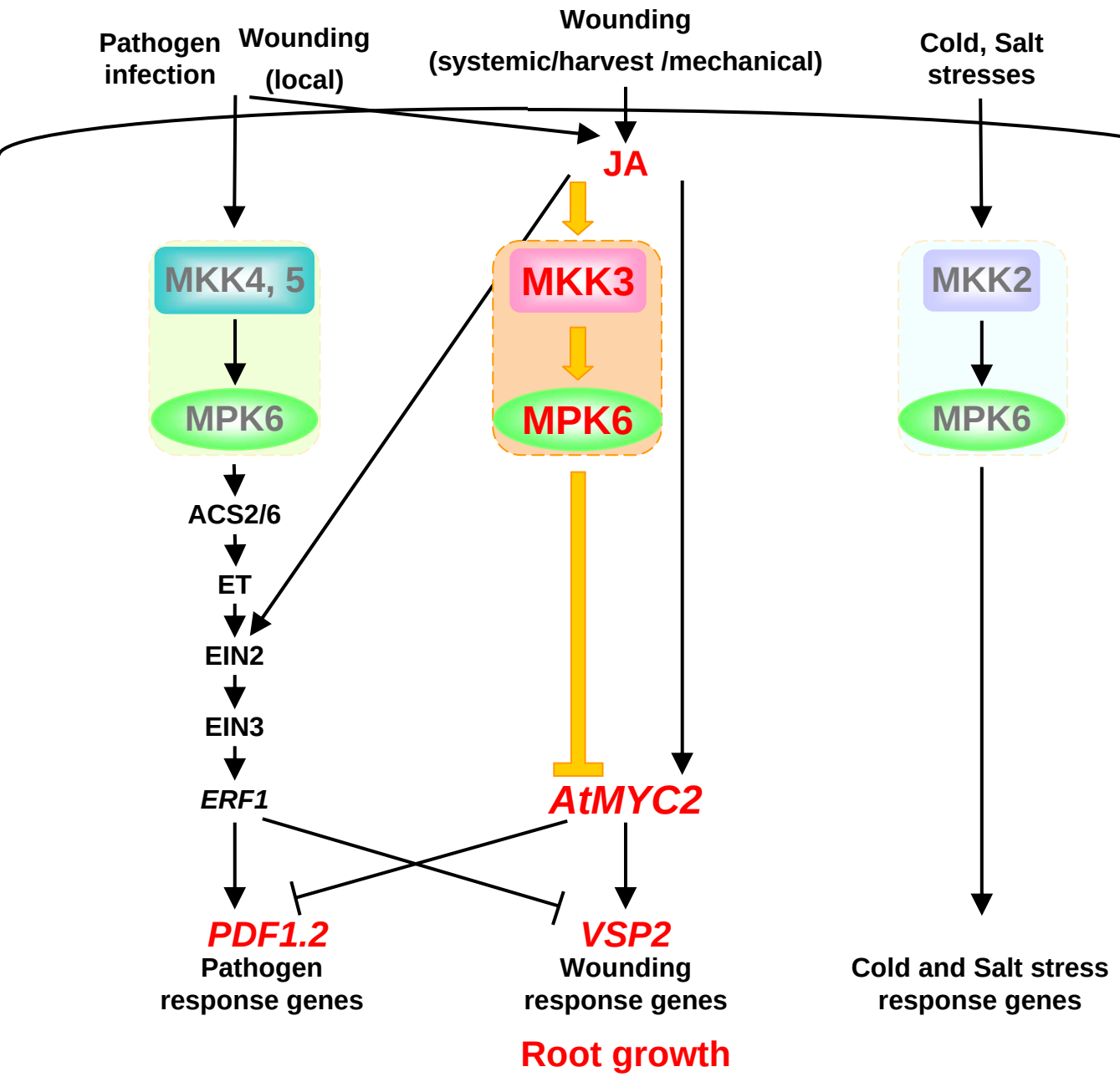

Figure 36. Possible roles of MPK6 signaling in *Arabidopsis*.
At least three cascades, MKK2–MPK6, MKK4/MKK5–MPK6, and MKK3–MPK6, function in *Arabidopsis*. Each plays a specific role in response to different stimuli, and the cascades transduce their signals to adapt to various environmental changes by cross talking with each other. The MKK2–MPK6 cascade regulates cold and salt stress-responsive genes, the MKK4/MKK5–MPK6 cascade regulates pathogen-responsive genes by activating ET biosynthesis, and the novel MKK3–MPK6 cascade regulates JA signaling. The JA-activated MKK3–MPK6 signal negatively regulates the JA pathway and affects JA-dependent gene expression and root growth sensitivity through *AtMYC2*.